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THE

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INTEGUMENTARY COLOR CHANGES IN THE NEWLY-BORN DOGFISH, MUSTELUS CANIS

G. H. PARKER

(*From the Biological Laboratories, Harvard University*)

INTRODUCTION

It is well-known that the integumentary melanophores of the lower vertebrates are functionally active at a very early stage. These color-cells become responsive at about the time young fishes hatch from their eggs. This is true of *Fundulus*, according to Bancroft (1912), Spaeth (1913), and Gilson (1926) and of *Coregonus* and of two species of *Salmo* according to Becher (1929). At this stage the melanophores begin to disperse or concentrate their pigment, thus giving to the young fish a dark or a light tint. Although the beginnings of larval life may be said thus to mark the period of the first color changes, Becher noted that the embryos of fishes on which he worked when artificially removed from their egg-shells would often show color changes. This demonstrated that melanophore activity is possible at least under experimental conditions earlier than the time of hatching. Such a view was also taken by Duspiva (1931), who worked upon two fishes, *Salmo salvelinus* and *Perca fluviatilis*. In *Perca* the first integumentary melanin appears five days after the eggs have been fertilized. Duspiva saw melanophore responses three days later or about eight days after fertilization. He believed, however, that responses may have occurred still earlier. As the young larvæ of *Perca* hatch from the eggs about ten days after fertilization, the first melanophore responses in this fish must occur in what is obviously its embryonic period. From all his observations Duspiva was led to conclude that melanophores probably become active as soon as their processes are developed and their melanin formed. Such a view, which appears to be fairly well supported, places the initiation of melanophore activity in fishes at a very early stage, not later than about the time of hatching and probably in some species somewhat earlier.

The first responses of melanophores in lower vertebrates were shown by Babák (1910) to be quite unlike those of later life. In the very young larvæ of the Mexican axolotl about 1.5 cm. long, when the first color changes had begun, the following conditions were found by Babák. In complete darkness the young animals were pale and in bright light they were dark. When they had attained a length of about 5 cm. their reactions in these respects were almost reversed. At this later stage they were in complete darkness dark and in bright light either pale or dark depending upon their surroundings. This second stage of their melanophore responses persisted until maturity when they became dark and remained permanently so, having lost their capacity to change. The early larval condition may be called the primary phase, the succeeding one the secondary phase. If a larva in the secondary phase is blinded, it was found by Babák to revert to the primary phase in that it would be pale in darkness and dark in bright light. A young larva in the primary phase when deprived of its eyes remained unchanged and was responsive in the same way as it had been before the operation. From these tests Babák concluded that what has been called here the primary phase was dependent upon the direct stimulation of the larval melanophores by light or its absence and that the secondary phase resulted from their indirect stimulation through the eyes of the larva. These conclusions were supported in one way or another by Pernitzsch's work (1913) on axolotl, by Hooker's work (1914) on *Rana pipiens*, and especially by an extended series of contributions from Laurens (1914, 1915, 1916, 1917) on the larvæ of several species of *Amblystoma*. Babák's views also received confirmation from the work of Fischel (1920) on various amphibians and especially from that of Duspiva (1931) on the two fishes already mentioned, *Salmo salvelinus* and *Perca fluviatilis*.

The references thus far cited pertain exclusively to oviparous fishes and amphibians. I know of only one writer who has recorded notes on the melanophore system of ovoviviparous vertebrates. In his paper on the genesis of chromatophores in fishes Eigenmann (1891) refers very briefly to the condition in the young of the ovoviviparous rockfish, *Sebastodes*, an embryo of which is figured by him. This small fish, when about to escape from the ovarian apparatus of the mother, is said by this author to have a very fully formed melanophore system whose functional possibilities, however, are not reported. Aside from this very meagre reference I know of no other on the melanophores in ovoviviparous vertebrates. It is therefore of interest to look into the condition of the newly-born pups of the smooth dogfish, *Mustelus canis* (Mitchill). This fish with its young can be had from time to time in the early summer at the Marine Biological Laboratory, Woods Hole, and I am under obligations to this Laboratory for the

material on which the following observations were made. I am also indebted to the Woods Hole Oceanographic Institution for the use of their equipment in working on these fishes. My studies were made under a grant given me from the Milton Fund of Harvard University for the investigation of color changes in marine animals. To the administrators of this Fund I wish to express my sincere thanks for the aid generously extended to me.

Observations

The smooth dogfish of the New England coast releases its young, four to a dozen or more at a time, in early summer. The following observations were made either on young dogfishes born in the laboratory tanks or on individuals removed from the females by what may be called a Cæsarean operation. After the envelopes and umbilical vessels of these young fishes had been severed they could be removed and carried gently to the sea-water tanks. During this procedure they were as a rule perfectly passive. After they had been immersed in the sea water of the tank for a fraction of a minute or so, normal respiratory gill movements began and at about the same time swimming was initiated. Such newly-born fishes swam for a short time with somewhat unsteady equilibrium, but they soon gained in steadiness and mingled with others of their kind in the tank. At the time of these tests in June the young fishes were about 25 cm. in length, the largest measuring 33 cm.

A young dogfish when first taken from the uterus of the female, a dark situation, is slightly dark in tint. This indicates that the young fish is in what has been called in this paper the secondary phase of melanophore activity, that is, the phase in which the fish is dark in darkness and pale or dark in the light according to the environment. Whether the dogfish passes through an earlier primary phase while it is still in the uterus of the mother is unknown. More likely this stage has been omitted in this fish as it appears to have been in the amphibians *Bombinator* and *Hyla* (Babák, 1910) and in the fish *Fundulus* (Bancroft, 1912; Spaeth, 1913; Wyman, 1924; Gilson, 1926).

Newly-born dogfishes respond in tint very quickly to that of their environment. On June 4 seven dogfishes were born in one of the experimental tanks. Five hours after their birth four were put in a white-walled, illuminated tank and three in a similar black-walled one. Two hours later those in the black-walled tank were extremely dark and those in the white-walled one decidedly light (Fig. 2). Preparations were made of the skin from an individual in each of these sets and microscopic views of the two preparations are shown in Figs. 3 and

4. As might be expected, the melanin in the color-cells from the pale dogfish is concentrated (Fig. 3), that from the dark one is dispersed (Fig. 4). On the following day the pale fishes were put into the black-walled tank and the dark ones into the white-walled one. Within two hours the fishes had reversed their tints, the pale ones having become dark and the dark ones pale.

In a second litter of dogfishes obtained by Cæsarean operation three were put immediately after their removal from the uterus into a black-walled illuminated tank and three into a white-walled one. The three fishes destined for the black tank were put into it at 9:17 in the morning. By 9:55 they had increased their dark tint. At 10:30 they were dark but not fully so, and at 10:50 they were fully dark. The three dogfishes intended for the white tank were put into it at 9:20 in the morning. By 9:55 they had lightened considerably but were still somewhat dark. By 10:30 they were fully blanched as compared with other young dogfishes used as checks. From these two sets of tests it is fair to conclude that specimens of *Mustelus* immediately after birth are capable of responding to the tint of their environment by appropriate melanophore reactions. In these responses they were quite like adults of their own species.

In another respect the young dogfishes also resembled adults. It has already been shown (Parker and Porter, 1934) that when a small transverse cut is made in the fin of a relatively dark *Mustelus* a pale band is soon formed extending from the cut toward the free edge of the fin. This band is believed to result from an excessive stimulation of the concentrating melanophoric nerve-fibers cut by the operation. Similar conditions obtain in the newly-born dogfishes. The pectoral fins of two such fishes were cut as described and 25 minutes after the operation both fishes had well-developed pectoral bands. One of these fishes was killed and a preparation of its pectoral fin was made. A photograph of this fin is reproduced in Fig. 1 and shows the typical pectoral bands.

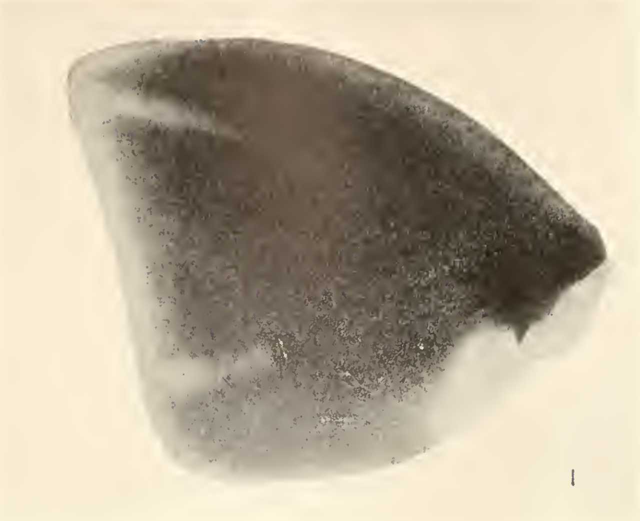
Explanation of Plate

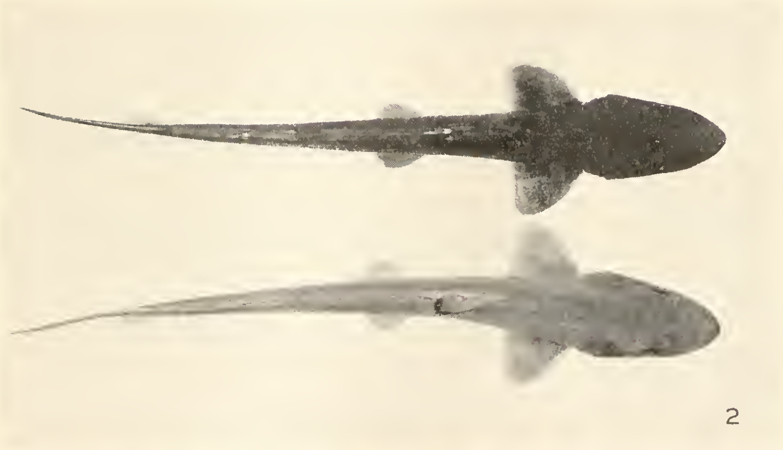
Fig. 1. Dorsal view of the left pectoral fin of a newly-born *Mustelus canis*. Two small cuts transverse to the fin rays have been made, the anterior one nearer the root of the fin, the posterior one nearer its lateral edge. A pale band extends from near each cut to the light edge of the fin.

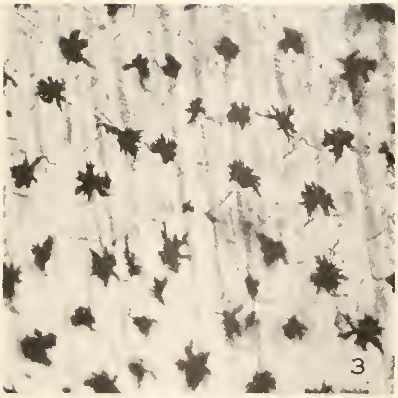
Fig. 2. Dorsal views of two newly-born *Mustelus canis*, the upper one in the dark condition, the lower one in the light condition. Preparations preserved in formaldehyde-alcohol.

Fig. 3. Microscopic view of the melanophores on the dorsal surface of the pectoral fin of a *Mustelus canis* in the light condition, melanin concentrated.

Fig. 4. A view similar to that shown in Fig. 3 of the fin of *Mustelus canis* in the dark condition, melanin dispersed.







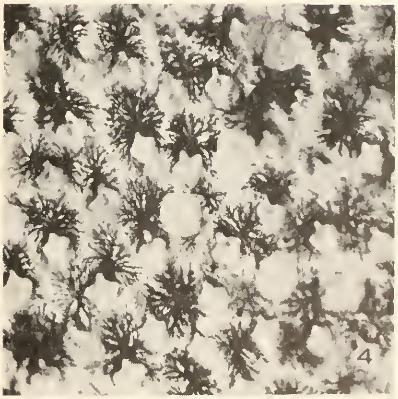


Plate I

In a third respect newly-born dogfishes resemble adults. They respond in the same way to adrenalin and to pituitrin (Parke, Davis and Company's preparations) as the mature dogfishes do (Lundstrom and Bard, 1932). If 0.2 cc. of a solution of adrenalin, one part in a thousand of water, is injected into a young dogfish of moderately dark tint, the animal will begin to blanch in about ten minutes and shortly after that it will assume a tint of extreme paleness. After two to three hours the young fish will reassume its darker tone. In a similar way an injection of 0.2 cc. of obstetrical pituitrin into a pale dogfish will induce its slow darkening. This change also passes away in about two hours after which the young dogfish will return to its former state. In both these respects the newly-born fishes resemble the adult.

These records show quite clearly that a young *Mustelus* immediately after birth possesses an active melanophore system whose reactions in a number of significant ways agree very fully with those of the adults. These ovoviviparous dogfishes then begin life in what has been called the secondary phase of melanophore activity. On the loss of their eyes they should lapse, according to the general theory of these relations, to the condition of the primary phase. Unfortunately my material was not sufficiently abundant to allow this test to be made.

SUMMARY

1. *Mustelus canis* is an ovoviviparous dogfish in which the young are born with a body length up to 33 cm.

2. At birth the young dogfishes are of a moderately dark melanophoric tint. This is doubtless the influence of the maternal body within which they have been lodged.

3. Immediately after birth these young dogfishes respond to their environment in that they change light or dark, conditions brought on by a concentration or a dispersion of their melanophore pigment.

4. Pale bands can be produced on the fins of newly-born *Mustelus* by cutting their nerves, as can be done with the adults.

5. A young *Mustelus* responds to injections of adrenalin by blanching and to pituitrin by darkening as adults do.

6. A newly-born *Mustelus* shows no evidence of the primary phase of color change seen in some other fishes and in some amphibians. It appears to omit this phase in its ontogeny and is born with a melanophore system that responds in the same way as this system does in the adults.

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LIGHT INTENSITY AND MELANOPHORE RESPONSE IN THE MINNOW, ERICYMBA BUCCATA COPE

F. A. BROWN, JR.

(*From the Zoölogical Laboratory of the University of Illinois*)[1]

Although every investigator of chromatic response in animals is well aware that light intensity has a profound effect upon the coloration of animals, this knowledge is at most qualitative and is in general restricted to the effects of the presence or absence of light. Fishes become pale in darkness as a rule while in light the response is governed by the color of the background. Considering that many animals with responsive pigmentary systems are normally accustomed to live at relatively low intensities of light, it was believed of significant value to investigate the behavior of fish from this viewpoint, and perhaps to derive simultaneously more information in regard to the sensitivity of the photoreceptors and the manner in which they function to produce adaptive color changes. In this latter regard Keeble and Gamble (1904), Bauer (1905), Sumner (1911), von Frisch (1911), Sumner and Keys (1929), Pearson (1930), and Sumner (1933) have concluded that the color of the animal is determined by the ratio of incident and reflected light striking the eye of the animal. Should this statement hold true without modification, then it would follow that for a given background which is characterized by always reflecting a given percentage of the light striking it, the animal would remain the same shade at all effective light intensities and there would exist a sharp critical point in intensity of light at which a fish upon a black background would become pale as the light was decreased. Whether or not this is the actual state of affairs is tested in the following experiment.

I. Uniform Black Background and Varying Light Intensity

Material and Methods

In the experiment herein described the silver-mouthed minnow, *Ericymba buccata* Cope, was used exclusively. This fish is from five to eight centimeters in length and is found most abundantly in streams with sand bottoms in the region about Urbana, Illinois, (Thompson and Hunt, 1930). The fish used were always freshly caught, none being used after they had been in the laboratory tanks for more than a week. Furthermore, all the data were obtained from fish caught in a given region and within a period of less than two winter months, thus insuring fish that were quite uniformly pigmented. Because of the rapidity of color change in this species no method was devised whereby more than a single light intensity could be used in testing the quantitative response in any individual fish. As a result, the data included here are those of a population and hence characterized by considerable variation.

---

[1] Contribution No. 474.

All the experiments were carried out in an experimental dark-room. The light sources used in obtaining the different intensities were a 200 watt tungsten lamp, a 75 watt daylight Mazda lamp, and a 25 watt tungsten lamp with a daylight filter. For the lower intensities the last lamp was enclosed in a light-proof container possessing an iris diaphragm over the single aperture. In addition, approximately neutral light filters (exposed photographic plates) were used. The measurement of the light intensity was made with a potassium photoelectric cell with maximum sensitivity in the blue end of the visible spectrum. This cell was constructed by Professor J. Kunz. In nearly every instance the light source was 125 centimeters directly above the 8 inch, flat-black-coated crystallizing dish in which the experimental fish were placed.

In order to test the effect of a given light intensity upon the response of the melanophores, two or three minnows were placed in the crystallizing dish containing three centimeters of water. The crystallizing dish was coated with flat-black paint because it was determined that upon a background of this nature the fish would become dark in color in the ordinary light intensities of the laboratory and would become pale in total darkness. Thus the actual intensity at which the fish would entirely cease to respond to the color of the background could be learned. At the end of two to three hours in the experimental situation, during which time the water in which the fish were kept was constantly aerated with compressed air and an aerator block, the fish were removed and immersed in boiling water for a minute or two. Then a piece of the integument from a given area of the fish was dissected off and mounted in glycerine. A Spencer camera lucida and a celluloid rule served in measuring the diameters of 30 to 80 melanin masses. The average was taken as an index of the degree of dispersion of the pigment in the fish as a whole. In order to ascertain that two hours was sufficiently long for the pigment masses to have reached an equilibrium point of dispersion within the melanophores for the light intensity and background in question, some of the fish were taken from a black background in light and others from a white background in light at the beginning of the experiment and thus the equilibrium points were reached from both sides. Furthermore, at the intensities of the ordinary daylight of the laboratory the color-change was very rapid, requiring only five or ten minutes to pass from one extreme to the other. Research is now in progress to determine to what extent the rates of color change are influenced by intensity.

Results

During the course of the experimentation 75 minnows were tested. These were roughly distributed among the seven light intensities in proportion to the variability exhibited in the data. Table I is a summary of the results.

TABLE I

Average diameters of melanin masses in fish kept upon a black background under measured light intensities. Pigment mass diameters are given in micra and light intensity in foot candles.

Light intensity						
.000005	.000053	.000625	.0125	.25	1.75	23.75
55.3	65.9	78.8	78.6	80.5	86.1	88.5
52.5	48.4	68.3	76.9	78.5	84.4	82.0
40.5	43.7	63.4	74.0	76.3	83.4	80.3
40.4	41.6	58.3	72.5	64.3	81.4	80.2
36.6	38.2	56.4	68.6	54.0	80.5	79.8
33.9	39.3	55.0	67.6	74.5	77.1	68.6
	36.3	51.9	59.6	73.6	77.0	66.7
	34.3	51.1	63.5	72.5	76.6	
	34.0	44.0	54.3	69.1	75.3	
	40.7	41.9	53.5	59.7	74.7	
	41.4	38.9	51.3	57.5	64.3	
		34.0	49.3	54.6		
			46.9			
			46.3			
			45.6			
			36.6			
Mean 43.1	43.4	53.4	59.2	67.9	78.3	78.0

The averages for a number of fish have been calculated for each light intensity. The values for individual fish are shown diagrammatically in Fig. 1, plotted against the logarithm of the light intensity. These data strongly suggest that the relationship is most probably one of a direct proportionality over the complete range of dispersion of pigment mechanically possible in the melanophore of this fish. In

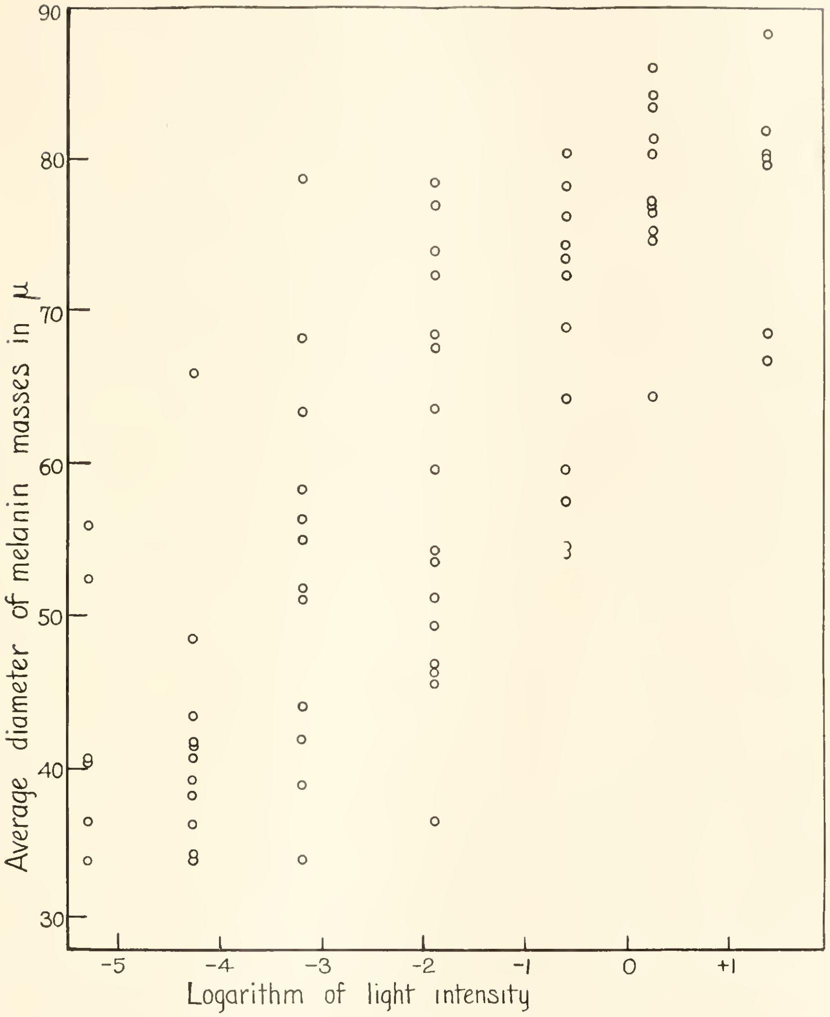

FIG. 1. Diagram showing the relationship between the logarithm of light intensity and the degree of melanin dispersion in the melanophores of *Ericymba buccata*. Each dot indicates the value for a single fish.

other words, the limits of this simple relationship are determined by the diameters of completely concentrated and fully dispersed pigment masses.

II. UNIFORM LIGHT SOURCE WITH DIFFERENT SHADES OF GRAY BACKGROUNDS

Material and Methods

A second type of experiment was performed using the same material and methods as in the first, except that here the light was kept constant at 1.75 foot candles, the minimum intensity at which complete dispersion of melanin could be obtained in response to a black background. Six backgrounds were used, the black one of the previous experiment, a white one, and four of intermediate shades of gray. The latter five were obtained by placing a glass crystallizing dish upon paper and cloth of the appropriate shades. The relative brightnesses of the direct light at the level of the background and the light reflected from the background were measured with a Macbeth illuminometer equipped with a brightness-determining device consisting of a translucent white plate. The values obtained serve best as comparative ones. The value of the ratio, $\frac{\text{direct light}}{\text{reflected light}}$, obtained for the white background was 2.54; for the gray backgrounds, 6.60, 12.4, 35.4, and 140.; and for the black one, 201.

TABLE II

Average diameters of melanin masses in fish kept upon black, white, and gray backgrounds in an incident light intensity of 1.75 foot candles. The ratio indicates the $\frac{\text{incident intensity}}{\text{reflected intensity}}$. Pigment mass diameter is given in micra.

Background ratio					
2.54	6.60	12.4	35.4	140	201
37.2	38.2	35.6	56.0	75.7	86.1
31.6	39.2	35.0	45.3	73.6	84.4
35.7	36.2	40.0	42.7	70.7	83.4
37.9			40.9	68.0	81.4
40.3			40.7	63.0	80.5
32.3			41.4	57.1	77.1
			46.4	55.7	77.0
			74.5	77.8	76.6
			59.5	74.8	75.3
			37.3		74.7
			38.2		64.3
			46.7		
			48.6		
Mean 35.8	37.9	36.9	47.5	68.6	78.3

Results

The average diameters of the melanin masses in 45 fish kept upon these above-described backgrounds are recorded in Table II. The values for the black background have been taken from Part I of this paper. These results plotted as a dot diagram in Fig. 2 indicate that

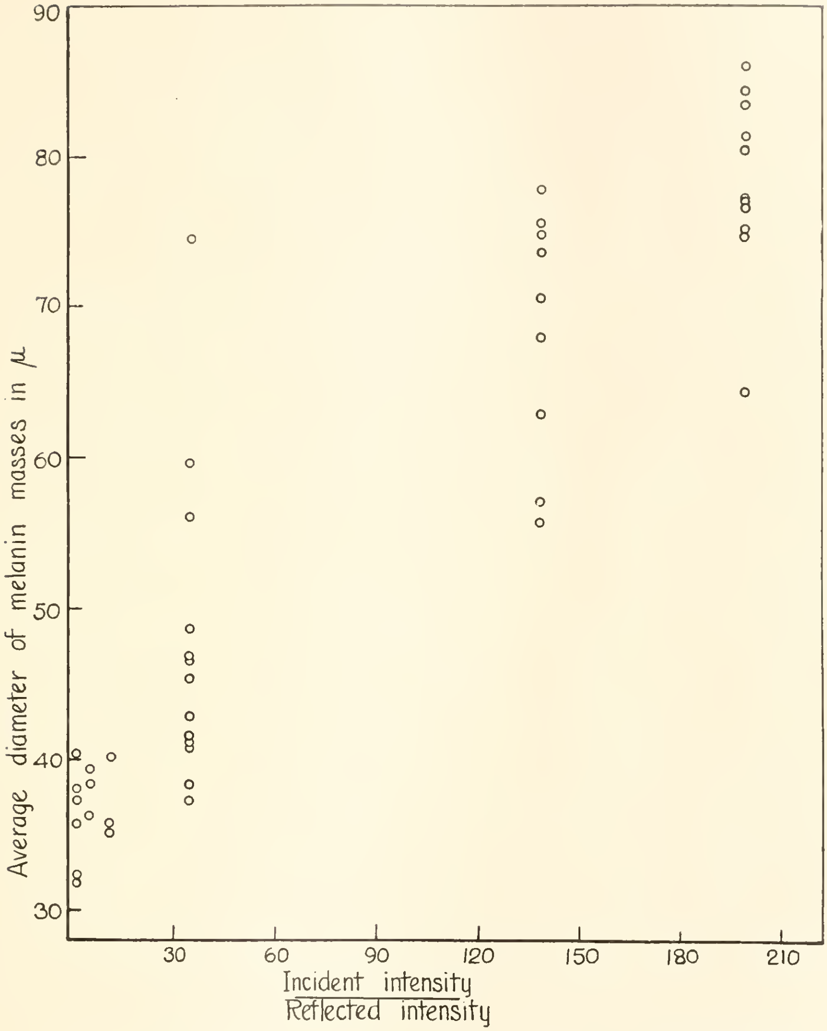

FIG. 2. Diagram showing the relationship between the ratio, $\frac{\text{incident light}}{\text{reflected light}}$, and the degree of melanin dispersion in the minnow, *Ericymba buccata*. Each dot indicates the value for a single fish.

for a given light intensity the average diameter of the pigment within the melanophore is a direct function of the ratio of incident to reflected light.

GENERAL CONSIDERATIONS

The ratio of incident to reflected light is thus seen to be inadequate to account completely for the degree of melanophore dispersion even when the intensity is considerably above the value of zero. Total intensity has a marked and significant effect. Duspiva (1931) found that light intensity had a very great effect upon the coloration of larvæ of *Perca fluviatilis*, *Salmo salvelinus*, and some other fishes. Background was without effect upon these forms. Koller (1934), however, reported that for larval *Coregonus* the background was the principal stimulus for melanophore activity while the light intensity had no influence until complete darkness was attained. These two papers are characteristic of the extremes in the literature regarding the effect of light intensity upon melanophores in fishes. The present research does not pretend to give a complete answer to this problem of long standing. Rather, it merely suggests that both forces are quite definitely influential in altering the coloration of fishes, but that the degree of overlapping of the two or the dominance of a single one determines the differences between individual species and even the same species at different developmental periods.

It is a well-known fact to fishermen of the Illinois River that when the water is laden with silt and consequently permits light to penetrate only a very short distance in any quantity, the fishes taken are pale in color, whereas in clear water the fishes are invariably dark in shade. If fishes swimming freely at some distance from the bottom in clear water can be figured as being upon the equivalent of a black background since practically no light is reflected from below, then the silt in the more turbid waters can be conceived of as having effect through reduction of incident light and also augmentation of the reflected light entering the eyes of the fishes.

In closing, I wish to acknowledge my indebtedness to Dr. David H. Thompson of the Illinois Natural History Survey and to Professor J. Kunz of the Physics Department, University of Illinois, for generously supplying me with helpful suggestions and material during this investigation.

SUMMARY

1. A quantitative method of determining the influence of the environment upon the melanophores of small fishes is described.

2. The degree of dispersion of the melanin in the melanophores of the silver-mouthed minnow, *Ericymba buccata*, is within certain limits determined by the total light intensity as well as by the shade of the background.

3. Upon a constant black background between the intensities of light, .000053 and 1.75 foot candles, the average diameter of pigment masses is directly proportional to the logarithm of the light intensity.

4. At an intensity of less than .000053 foot candles the fish are at their maximum degree of paleness in spite of a black background.

5. At 1.75 foot candles illumination the fish becomes maximally dark upon a black background, and the average diameter of melanin masses appears to vary in a directly proportional fashion with the ratio, $\frac{\text{incident light}}{\text{reflected light}}$, which reaches the eyes of the fish.

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RENAL FUNCTION IN MARINE TELEOSTS

II. The Nitrogenous Constituents of the Urine of Sculpin and Flounder, with Particular Reference to Trimethylamine Oxide

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In view of the rather extensive work which has been done upon kidney function in the sculpin (*Myoxocephalus octodecimspinosus*), a common marine teleost (for review of literature see 34), it became of interest to investigate more fully the nitrogenous constituents of the urine. For comparison the flounder (*Pseudopleuronectes americanus*) was likewise studied. Analysis of samples of urine from these two species for the ordinary nitrogenous constituents showed that approximately one-half of the total nitrogen was still unaccounted for. The presence of trimethylamine oxide in other marine fishes (see Table III) made it appear likely that this compound might be present in the urine of our animals. Investigation has demonstrated its occurrence in sculpin urine in significant amounts, but its absence in detectable quantities from the urine of the flounder. Brief supplemental studies demonstrated the occurrence of the oxide in the urine of the daddy sculpin (*Myoxocephalus scorpius*) and confirmed its presence in the urine of the goosefish (*Lophius piscatorius*) (18).

Material and Methods

Freshly caught sculpins and flounders were brought into the laboratory, the urinary papilla was tied off, and the fish were transferred to live cars, usually for 24 hours, sometimes longer. At the end of this time they were killed by a blow on the head, and the urine was removed from the exposed bladder by syringe. Following are additional details concerning the urine samples listed in Tables I and II; unless otherwise noted preservation was by toluol and hydrochloric acid. All samples were kept in a refrigerator when not in use. *Sculpin 1:* about 475 cc., from 217 sculpins; chloroform and toluol. *Sculpin 2:* about 475 cc., from 239 sculpins; 1 per cent sulfuric acid. *Sculpin 3:* 15 cc. from a few specimens. *Flounder 1:* about 475 cc., from 135 flounders; chloroform and toluol. *Flounder 2:* about 475 cc., from 117 flounders; 1 per cent sulfuric acid. *Flounder 3:* 15 cc. from a few specimens. *Flounder 4:* same. *Daddy sculpin:* from two specimens. *Goosefish:* from one specimen in good condition. All sculpins and all but one group of fifty flounders (taken in net) were caught with hook and line. The urine samples were collected at the Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine.

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[1] With the assistance of Gordon Spence.

Total nitrogen was determined by the methods of Van Slyke (49) (*Sculpin 1* and *2*, *Flounder 1* and *2*) and Pregl (39) (all other specimens); ammonia by the method of Folin and Bell (13); urea by the method of Van Slyke (50), using 0.5 M. veronal rather than phosphate as a buffer because of the high magnesium content of the urine; uric acid by the method of Benedict and Franke (4); amino acids by the method of Folin (12); total creatinine by the method of Folin (11); and chloride by the method of Van Slyke (48) as modified by Smith (41). Trimethylamine oxide was determined by the method of Hoppe-Seyler (24).

RESULTS

The analytical data for the ordinary nitrogenous constituents of the large pooled samples of sculpin and flounder urine are summarized in Table I. The chloride values for these samples, in millimols per liter, are as follows: *Sculpin 1*, 99; *Sculpin 2*, 105; *Flounder 1*, 184; *Flounder 2*, 138. It has previously been demonstrated (15) that sculpins, when handled under rather ideal conditions, with marked precautions to avoid skin injury, regularly show a higher average urinary total nitrogen and a lower average urinary chloride (see also 16) than those reported here. With repeated handling under the usual experimental conditions, the animals typically develop a diuresis, which is characterized by a rather rapid decrease in urinary con-

TABLE I

Distribution of nitrogenous constituents of sculpin and flounder urine.

	Sculpin 1		Sculpin 2		Flounder 1		Flounder 2	
	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen	Nitrogen	Total nitrogen
	mgm./100 cc.	*per cent*	*mgm./100 cc.*	*per cent*	*mgm./100 cc.*	*per cent*	*mgm./100 cc.*	*per cent*
Total N	88.0		84.5		43.4		84.0	
Urea N	13.5	15.3	11.9	14.1	9.2	21.2	11.3	13.5
Ammonia N	1.1	1.3	1.7	2.0	0.8	1.8	2.2	2.6
Uric acid N	0.6	0.7	0.6	0.7	0.5	1.2	1.1	1.3
Amino acid N	3.9	4.4	3.4	4.0	4.2	9.7	6.9	8.2
Total creatinine N	19.7	22.4	21.1	25.0	6.5	15.0	21.9	26.1
Undetermined N	49.2	55.9	45.8	54.2	22.2	51.1	40.6	48.3

centration of total nitrogen and a sharp increase in the urinary concentration of chloride (15, 37). Under the circumstances, the divergence in the present samples from the more ideal values can undoubtedly be attributed to the manner in which the sculpins were handled. With the large number of animals used, the handling was necessarily rather rough as compared to the rigid technic employed in more exacting experiments (15, 16). This also applies to the total nitrogen value given for the daddy sculpin (Table II). From urinary chloride data upon flounder presented elsewhere (16), the high chloride and low total nitrogen values in the samples of flounder urine can be similarly interpreted. In line with this interpretation is the fact that specimen *Flounder 1*, containing urine from a batch of fifty flounders subjected to rather severe conditions and overcrowding in a flounder net, shows a very low total nitrogen and a very high chloride concentration. The total nitrogen content of the sample of goosefish urine (Table II) is well below the values reported by Grollman (18) for bladder urine from freshly caught specimens, is comparable with those given by Edwards and Condorelli (8), and is far above those reported by Marshall and Grafflin (35) and Smith (40), whose specimens were obviously quite diuretic.

The abnormalities in urine formation observed under experimental conditions are associated primarily with a derangement of the normal salt and water balance of the organism, resulting from the loss of water through the skin and the increased ingestion and gastro-intestinal absorption of sea water (42, 17). The important fact, for the present data, is that from all available evidence there is no concomitant derangement of nitrogen metabolism under these conditions; i.e., the decreased urinary concentration of total nitrogen represents primarily a dilution effect. This statement is borne out by a comparison of the nitrogen distribution data upon the urine of the goosefish previously reported by Grollman (18) and Smith (40), the former at high and the latter at low urinary total nitrogen concentration. Under the circumstances, the analytical data given in Table I are interpreted as representing fairly accurately the normal percentage distribution of the ordinary nitrogenous constituents and percentage of undetermined nitrogen in sculpin and flounder urine.

The ammonia and uric acid values for sculpin and flounder are quite low, and are comparable with those reported by Grollman (18) for *Lophius*, but much lower than those reported for ammonia in *Lophius* and *Muræna* by Edwards and Condorelli (8). Urea is present in considerable amounts in sculpin and flounder urine, some data on sculpin having been reported by Marshall and Grafflin (36). The urinary excretion of urea in significant amounts seems to be characteristic of all marine teleosts so far studied except the goosefish (*Lophius piscatorius*). Marshall and Grafflin (35) reported that the plasma and urine of this species uniformly contain urea in only minimal amounts, and this was subsequently confirmed for the urine by Grollman (18) and Smith (40). The high values reported for *Lophius* by Edwards and Condorelli (8) seem to cast doubt upon all of their urea analyses. The amino-acid and total creatinine values for sculpin and flounder are to be compared with other data available in the literature (8, 18, 35, 37, 40). In the marine teleosts as a group, the total creatinine fraction of the urine consists predominantly of creatine. Fairly extensive data upon the creatine content of sculpin urine, under normal and diuretic conditions, have been supplied by Pitts (37).

It is evident from Table I that approximately one-half of the total nitrogen in the composite samples of sculpin and flounder urine is unaccounted for by the ordinary nitrogenous constituents. The demonstrated presence of trimethylamine oxide—$(CH_3)_3NO$—in the muscles of marine elasmobranchs and teleosts and particularly its presence in the urine of the goosefish (see Table III) made it appear likely that this compound might be present in the urine of our animals. Using the micro-method of Hoppe-Seyler (24), results were obtained which indicated the presence of the oxide in considerable amounts in sculpin urine, but its absence in readily detectable quantities from flounder urine. Since there was a rather long interval between the collection of these urine samples and their analysis for trimethylamine oxide, and since the oxide has been found to undergo a slow decomposition to trimethylamine even in well-preserved urine (18), these data were rejected and new urine samples were collected in the following summer. These samples were analyzed soon after collection (Table II) and confirmed the previous findings. The $(CH_3)_3NO$ figure (22.8) for the fresh composite sample of sculpin urine (*Sculpin 3*)

TABLE II

Trimethylamine oxide in urine of sculpin, flounder, daddy sculpin and goosefish.

	Sculpin 3	Flounder 3	Flounder 4	Daddy sculpin	Goosefish
Total N, *mgm. per cent*	98.5	66.2	65.8	44.5	212.0
$(CH_3)_3NO$ N, *mgm. per cent*	22.8	−0.3	1.4	14.1	69.1
Total N, *per cent*	23.1	—	—	31.7	32.6

is to be compared with the figure 20.4 (milligrams per cent of nitrogen as $(CH_3)_3NO$) obtained upon sample *Sculpin 2* (preserved with sulfuric acid) more than two months after collection. This latter value represents 24.1 per cent of the total nitrogen, 44.5 per cent of the undetermined nitrogen. Although present in sculpin urine in considerable amounts, trimethylamine oxide is apparently entirely absent from flounder urine, or present in only small traces. The amount present in the urine of the daddy sculpin is comparable with that in the sculpin; and the value for goosefish urine is essentially confirmatory of the results of Grollman (18).

In order to obtain additional evidence that the substance present in sculpin urine is actually trimethylamine oxide, this compound was isolated from urine sample *Sculpin 1* as the aurichloride. For trimethylamine oxide this salt would have the composition $(CH_3)_3NO \cdot HAuCl_4$. Synthetic material of this composition gave a melting point of 255–7°, which agreed exactly with that of the salt isolated from sculpin urine. The mixed melting point was likewise 255–7°. On analysis the sculpin salt gave the percentage of gold as 46.0, 46.25 (theoretical 47.5 per cent); percentage of nitrogen 3.5 (theoretical 3.4 per cent). From the aurichloride the picrate was prepared in the following manner. The gold salt was dissolved in water and treated with "molecular" silver until the yellow solution became colorless. The silver was then centrifuged off and slightly less than the calculated amount of picric acid solution was added. The melting point of the picrate from the sculpin gold salt and of synthetic picrate from trimethylamine oxide, as well as the mixed melting point, all agreed: 194–7°. In addition, the picrates from the natural and synthetic sources had the same crystalline form.

Thus there is adequate evidence that trimethylamine oxide makes up a considerable portion of the "undetermined" nitrogen fraction of sculpin urine. As to the nature of the compounds present in the remaining fraction of the undetermined nitrogen in this species, we have no satisfactory data. This applies also to the entire undetermined nitrogen fraction of flounder urine.

DISCUSSION

The presence of trimethylamine oxide and trimethylamine in the fishes has been the subject of considerable investigation, and the present state of our knowledge is summarized in Table III. Supplemental to the table is the clear demonstration of the presence of trimethylamine oxide in the blood of *Scyllium catulus* (24) and in the roe of *Clupea harengus* (38).[2] It is obvious that our negative finding for the urine of the flounder, *Pseudopleuronectes americanus*, is particularly striking in view of the positive results obtained from the muscles or urine of all other marine teleosts so far examined, especially from the muscles of the closely related *Pleuronectes cynoglossus*. The generalization has frequently been made that trimethylamine oxide is characteristic of marine, as opposed to fresh water, teleosts. Its absence from the urine of the flounder does not, naturally, preclude the possibility of its occurrence in the muscles of this species.

TABLE III

The occurrence of trimethylamine oxide and trimethylamine in the muscles and urine of fishes.

	Muscles		Urine		References
	TrO	Tr	TrO	Tr	
Selachians (Marine)					
Acanthias vulgaris	+	+	+	−	21, 24, 43, 44
Lamna cornubica	+	+	−	−	21
Scyllium catulus	−	−	+	+	24
Teleosts					
a. Marine					
Clupea harengus	+	+	−	−	21, 38
Gadus æglefinus	+	+	−	−	21, 25
Gadus morrhua	+	+	−	−	21, 25, 26
Mullus barbatus	+	+	−	−	21, 25
Pleuronectes cynoglossus	+	+	−	−	21, 25
Pseudopleuronectes americanus	−	−	0	−	This paper
Conger vulgaris	+	+	−	−	21, 25
Lophius piscatorius	−	−	+	+	18, this paper
Myoxocephalus octodecimspinosus	−	−	+	−	This paper
Myoxocephalus scorpius	−	−	+	−	This paper
b. Fresh water					
Anguilla vulgaris	0	0	−	−	21, 25
Salmo salar	0	0	−	−	21, 25
Squalius cephalus	0	0	−	−	21, 25
Alburnus lucidus	0	0	−	−	21, 25
Perca fluviatilis	0	0	−	−	21, 25
Cyprinus carpio	0	0	0	0	21, 24

+ = present; 0 = absent; − = untested; TrO = trimethylamine oxide; Tr = trimethylamine.

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[2] Grollman (18) concludes that the trimethylamine present in goosefish urine was probably absent from the fresh specimens, and resulted from the slow decomposition of the oxide; and Hoppe-Seyler (24) indicates the possibility of a similar origin for the amine present in his samples of selachian urine. He further states (23, 24) that one cannot for the present be certain that the small amounts of trimethylamine previously (21) found in extracts of fresh muscles of selachians and marine teleosts (and originally regarded as a metabolic rather than a decomposition product) do not arise from the oxide in the course of the working-up process. He could also detect no relationship between the presence of the amine and its oxide and seasonal variations or the spawning period.

Trimethylamine oxide has further been demonstrated in cephalopods (20) and, on the basis of Hoppe-Seyler's interpretation (22), in crustacea by Suzuki *et al.* (45, 46), who reported it as "kanirin." [3] From the gonads of the jellyfish, *Rhizostoma cuvieri*, Haurowitz (19) has isolated trimethylamine, which is accepted as existing preformed in the tissues (21). Neither the amine nor its oxide has ever been reported as occurring in the normal tissues or body fluids of amphibia, reptiles or birds. Kappeler-Adler and Krael (26) reported an indirect demonstration of the oxide in mammalian muscle, and Lintzel (32) has very recently claimed trimethylamine and trimethylamine oxide to be normal constituents of human urine.[4]

From a detailed consideration of the osmotic relationships existing between the fishes and their environment, Hoppe-Seyler (23, 24) concludes that the retention of nitrogenous metabolic products creates the conditions which are necessary for the formation of the oxide. Thus the selachians, which are able to hold the salt content of their body fluids much lower than that of sea water, establish isotonicity with their environment through the retention of urea, trimethylamine oxide and betaine, and thus hinder the withdrawal of water from the body by the surrounding medium. Actually, Hoppe-Seyler's conception of the existing relationships in marine elasmobranchs is erroneous. Selachian blood is hypertonic to sea water, and this group is to be classed along with fresh water fishes as osmotically superior to the environment (42). That there is an active conservation of the oxide by the kidneys has been established by his demonstration of the much lower concentration in the urine as compared with the blood. According to Hoppe-Seyler, in the marine teleosts a similar, but much smaller, retention of metabolic end-products must occur, and in this way the difference between the osmotic pressure of the sea water and of the blood is at least diminished. The validity of this argument has been questioned by Homer Smith, in an article to appear in *Biological Reviews*, on the ground that direct evidence for an active retention in marine teleosts has never been adduced. On the other hand, in fresh water teleosts, living in a markedly hypotonic medium, the conditions are such that metabolic products are withheld as little as possible, and the oxide never occurs (Table III).[5] Hoppe-Seyler extends his argument to the presence of the oxide in cephalopods and of the betaines in marine invertebrates and in plants, and makes the inclusive generalization that wherever nitrogenous metabolic products are retained, the preliminary conditions for the occurrence of methylation processes are present.

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[3] The closely related tetramethylammonium hydroxide has been isolated from *Actinia equina* (2).

[4] The presence of trimethylamine in human urine has often been reported (among others, 5, 10, 14); but following the investigations of Takeda (47), Kinoshita (27) and Erdmann (9) it has been generally accepted that the amine does not exist preformed in the urine, but is liberated from some precursor by either chemical or bacterial decomposition. Lintzel refuses to accept this conclusion, and goes on to show that when large quantities of trimethylamine oxide, betaine, choline, etc. are added to human urine, the yield of trimethylamine does not increase. He further reports that when urine is distilled for trimethylamine for several hours, a subsequent distillation yields no more of the substance, even though a considerable quantity of trimethylammonium base is present. Actually, Takeda concluded that preformed trimethylamine is perhaps present at times in normal human urine, though always absent from that of the dog and horse. Trimethylamine has likewise been reported in human cerebrospinal fluid (7) and in the blood of lower mammals (6, 7) and man (7, 14). However, these reports have all been subjected to the same criticism that the amine may well have arisen from some precursor through chemical or bacterial decomposition.

The conclusion of this investigator (21, 23), that on the basis of all available data trimethylamine and trimethylamine oxide must be considered primarily as end-products of protein metabolism, is concurred with by Poller and Linneweh (38) and Grollman (18), and is confirmed by the demonstration of the oxide in the urine of selachians and marine teleosts (23). This is in agreement with the general view expressed by Kutscher and Ackermann (29, 30) for the methylated muscle bases. Hoppe-Seyler (21, 23) regards trimethylamine and its oxide as derived primarily from ammonia through methylation and oxidation; and sees in the formation of the oxide the replacement of ammonia, which is toxic for the vertebrates in high concentration, by an essentially non-toxic, very weakly basic compound. The other methylated muscle bases, on the other hand, most probably are derived from higher degradation products of proteins, the amino acids (30). The idea that trimethylamine and its oxide may arise from the betaines, cholines (lecithin) and other compounds has also been advanced (18, 21, 28), and Lintzel (32) accepts such an origin in the human through the activity of the intestinal bacteria (see below).

The possibility that trimethylamine and its oxide are, in the fishes, in part at least of exogenous origin, being ingested preformed, has been accepted by Grollman (18) and Hoppe-Seyler (21). The latter, however, believes it impossible that this could form the only source for the amounts demonstrable in selachians and marine teleosts, even though one assumed a strong retention of nitrogenous metabolic products. Lintzel (32) similarly accepts an exogenous origin for part of the trimethylamine and trimethylamine oxide in human urine. According to the latter investigator, ingested trimethylamine would appear in the urine principally as the oxide.

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[5] Hoppe-Seyler (23) sought to settle the question whether trimethylamine and its oxide actually do appear as metabolic products in fresh water teleosts, but are not demonstrable because of their rapid and complete excretion in the dilute urine. Carp were allowed to swim about in limited quantities of water over a period of seven days, and the entire body of water (containing the urine), when concentrated to small volume and treated with zinc dust and HCl, gave no precipitate with gold chloride. The logical conclusion was that the amine and its oxide are not formed in fresh water fish. Smith (40) reported a volatile base, in addition to ammonia, in the branchial and urinary excretions of the carp, and concluded that it was presumably in large part trimethylamine liberated from the oxide on treatment with zinc dust and alkali. The inability of Hoppe-Seyler to detect either the oxide or the free amine in the urine or muscles of this species (21, 24) renders Smith's interpretation untenable.

Hoppe-Seyler (21) has concluded that one must consider the possibility that in the fishes trimethylamine and its oxide can, at least in part and under certain conditions, be demethylated. From the work of Löffler (33), who showed an increase in urea formation when the surviving liver was perfused with trimethylamine, it is reasonable to assume that demethylation is a normal process in the mammals.

Suwa (44) first showed that in the presence of putrefying tissue trimethylamine oxide was converted to the free amine; and Poller and Linneweh (38), on the basis of their demonstration of the oxide in the muscle of *Clupea harengus* and this known bacterial conversion, offered an explanation for the large amounts of trimethylamine known to be present in herring brine. Ackermann and Schütze (1) and Kohlrausch (28) demonstrated the liberation of the amine from lecithin, choline and betaine under bacterial influence; and Takeda (47) demonstrated the appearance of trimethylamine in considerable amounts in mammalian urine allowed to undergo ammoniacal fermentation. Later, Ackermann, Poller and Linneweh (3) showed that the conversion of trimethylamine oxide to the amine, demonstrated by Suwa with putrefying tissue, could also be effected by fresh liver, even when cooked, indicating in the latter case a non-fermentative reductive mechanism. Lintzel (32) discusses the assumption that a bacterial decomposition of some of the trimethylammonium bases occurs in higher organisms; and concludes, on the basis of extensive feeding experiments with betaine, choline, lecithin, γ-butyrobetaine, carnitine and meat extract, that trimethylamine and its oxide, insofar as they are not ingested preformed, must owe their presence in normal human urine to the activity of intestinal bacteria upon certain of these bases, liberating trimethylamine, which would then appear in the urine for the most part as the oxide (see below).

In feeding experiments on human beings, Lintzel obtained the surprising result that ingested trimethylamine appears almost quantitatively (within 10 per cent) as trimethylamine oxide in the urine, and concludes that we must designate the oxide as the normal end-product of trimethylamine metabolism in man.[6, 7] On feeding preformed trimethylamine oxide (both as the pure compound and as marine teleostean muscle), he found the majority of it excreted unchanged in the urine, with a moderate rise in trimethylamine.[8] He commented upon the rough proportionality between the oxide and the free amine in the human as indicating some sort of physiological relationship, perhaps with respect to oxidative-reductive processes in the organism; and cited in support of this idea the *in vitro* experiments of Ackermann, Poller and Linneweh (3), who stressed the biological importance of the oxide as a hydrogen-acceptor, particularly with respect to the sulfhydryl group of reduced glutathione.

It is a pleasure to acknowledge the capable assistance of Gordon Spence, who accepted full responsibility for the laborious task of catching, handling and collection of urine from the sculpins and flounders used for Specimens 1 and 2.

SUMMARY

It has been shown that approximately half of the total nitrogen in the urine of the sculpin (*Myoxocephalus octodecimspinosus*) and the flounder (*Pseudopleuronectes americanus*) cannot be accounted for by the ordinary nitrogenous constituents. Trimethylamine oxide is present, and accounts for a large portion of this unknown fraction in the sculpin, but is either absent or present in only very small quantities in the urine of the flounder. Brief supplemental studies demonstrated the presence of trimethylamine oxide, in considerable amount, in the urine of the daddy sculpin (*M. scorpius*) and confirmed its presence in the urine of the goosefish (*Lophius piscatorius*). In the discussion the literature concerning the occurrence and significance of trimethylamine oxide and trimethylamine is reviewed.

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[6] Interesting in this regard is the finding (24) that when trimethylamine was injected into carp (in which neither the amine nor its oxide normally occurs) it could be recovered in part from the urine, but that no trimethylamine oxide was formed.

[7] Langley (31) had previously claimed that rabbits metabolized the large majority of ingested trimethylamine, the remainder being excreted unchanged in the urine. He further concluded that part, if not all, of the metabolized amine seemed to be excreted as urea.

[8] Suwa (44) obtained essentially similar results on feeding experiments with rabbits. When the oxide was injected subcutaneously, on the other hand, it was excreted in part as trimethyl- and dimethylamine, but he could detect no unchanged oxide in the urine.

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THE EFFECT OF X-RAYS ON CLEAVAGE IN ARBACIA EGGS: EVIDENCE OF NUCLEAR CONTROL OF DIVISION RATE

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There has been much speculation as to what part of the cell, after exposure to short wave-length radiation, is most responsible for bringing about certain observable biological changes—changes in rate of growth, rate of mitosis, and the like. Also, there has been much speculation as to the influence of the nucleus, if any, on the rate of cell division. The present investigation gives information on these seemingly unrelated questions.

In previous reports (Henshaw, 1932, 1933) we have described the effects of X-rays on the time of the first cleavage in the eggs of *Arbacia punctulata* when irradiated before fertilization. At this time we wish to supplement this by describing the effects when sperm alone are irradiated, and also, when both eggs and sperm are irradiated. As a background for the present work the conditions and findings of the previous work will be reviewed briefly.

Arbacia eggs can be collected in large numbers from a single female; likewise, sperm can be collected in large quantities from a single male. If arranged properly in sea water, the eggs may be fertilized with the sperm and normal development observed. If a few drops of sperm suspension are added to a sample of eggs and the time for cleavage noted, the first cleavage will be found to have occurred in half of the eggs in about 55 minutes under ordinary conditions. If these conditions are kept the same for various samples of a given collection, the cleavage time (i.e., the time from the moment of insemination to the moment when 50 per cent have cleaved) will be constant within one minute ordinarily.

If, however, the eggs are exposed to X-rays immediately before fertilization, the onset of the first cleavage is noticeably delayed. Thus, the radiation in some way affects the protoplasm of the eggs and as a result the first cellular division is slowed. If graded doses of radiation are given to a series of samples, the amount of delay will be found to increase as the dose of radiation increases. In the present study the same irradiation effect (cleavage delay) has been produced when sperm alone were irradiated. Because sperm and eggs differ distinctly in certain respects, it has been possible to obtain some indication of what part of the cell is affected by the radiation in bringing about cleavage delay, and also something of the relation of cell parts to cell function.

Procedure and Results

Arbacia were collected during the spawning season and maintained in running sea water until needed for use. When material was required, the ovaries were removed from a single female and allowed to shed eggs into a large volume of sea water (several hundred ml.). The ovarian tissue was removed by filtering the suspension through cheesecloth and the eggs were then allowed to settle. Three drops of heavy egg suspension were placed in each of several small glass vessels and the material was then ready for treatment. The vessels had flat bottoms and were about 8 mm. in diameter. The egg suspension was about 2 to 3 mm. deep in the vessels.

Testes were removed from a single male and minced slightly with fine pointed forceps. Portions of the resulting material were then placed in other glass vessels of the same design to a depth of 2 to 3 mm. and were thus ready for treatment.

By laying out the work properly, it was possible to obtain data for three types of experiments at the same time on different portions of the same collection of material—data for (1) irradiated eggs and normal sperm; (2) irradiated eggs and irradiated sperm; and (3) normal eggs and irradiated sperm. It was necessary, therefore, to have on hand both irradiated and non-irradiated material. Accordingly, some of the vessels of sperm and eggs were not treated. The samples receiving treatment were arranged in a special way for exposure to the radiation. Since the time factor has been shown previously to have an influence on the results obtained, the same duration of exposure (30 minutes) was given to all samples. Varying doses of radiation were obtained by placing the samples at varying distances from the source of radiation.

One sample each of sperm and eggs was placed at the distances of 20, 28.3, 40 and 56.5 cm. from the center of the target of the X-ray tube. These distances were selected in accordance with the Inverse Square Law, reducing the intensity by one half in each case. Since means were not available for measuring the intensity at each distance, the relative dosages must be regarded as only approximate. This, however, does not interfere with the relative effects obtained on sperm and eggs, which is the main point of interest here. The intensity at

20 cm. distance was approximately 720 rœntgens per minute, knowledge of the exact amount not being essential. The radiation was supplied by a standard, Coolidge Type, air-cooled, tungsten target, X-ray tube. The conditions under which the radiation was obtained were 120 kv., 180 pv., 5 ma. and a filter of thin paper.

After exposure the eggs were arranged in Syracuse dishes in a larger volume of water and sperm suspension in proper dilution was added to make the combinations mentioned. Insemination of the different samples was accomplished as nearly simultaneously as possible, never more than two minutes being required for this. Determination of the cleavage time was then carried out according to the procedure described previously.

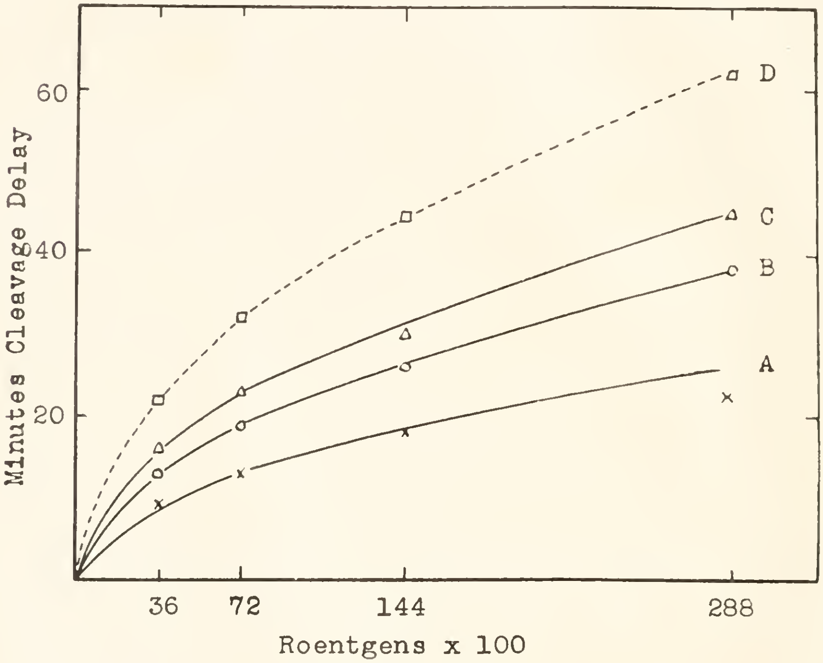

FIG. 1. Curves showing how irradiation effect (cleavage delay) varied with respect to dose of radiation administered. Curves *A* and *B* show the results obtained when eggs and sperm, respectively, were irradiated, while Curve *C* shows it when both eggs and sperm were irradiated. Curve *D* shows the effect which would have been obtained if the effects produced in sperm and eggs had been entirely additive.

A series of nine experiments have been carried out, all of which show essentially the same thing. Averaged results for three of these, carried out under precisely the same conditions, are shown in Fig. 1.

The abscissæ indicate dose in rœntgens, while the ordinates indicate irradiation effect in minutes cleavage delay. Curve *A* shows the effects obtained when eggs alone were given increasing amounts of radiation. This, in general, is like the curves obtained previously for similar conditions. Curve *B* shows the effect when sperm alone were irradiated and Curve *C* shows it when both sperm and eggs were irradiated.

It will be seen, not only that cleavage delay is produced by the irradiation of sperm alone, but also that the amount of delay is greater for a given dose when sperm are irradiated than when eggs are irradiated. While the actual amount of difference varied somewhat from experiment to experiment, the ratio of difference obtained for the two gametes remained approximately the same. Also, it will be seen that still greater effects are obtained when both eggs and sperm are irradiated. This indicates that the effects are to some extent additive when both elements are treated.

Discussion

The significance of this investigation may best be considered by analyzing step by step the processes involved. Experiments with irradiated eggs will be taken up first.

An affecting agent, radiation, was administered to a certain type of cell, the *Arbacia* egg, and changes of some kind were produced. These were made evident, at least in part, by the addition of sperm and the determination of the time required for cleavage. The sperm in such a case does two things of consequence. First, it enters the egg and incites it to division, thus initiating the reaction by which the irradiation effect is detected and measured. Second, it carries into the egg a portion of protoplasm which has not been irradiated. Hence, since radiation was allowed to affect only the egg, the procedure of adding sperm and timing cleavage may be regarded mainly as a means used in detecting irradiation effect. Changes produced in the egg, therefore, must have been responsible for the slowing of division observed.

When the same effect is obtained by irradiating sperm, quite a different problem is presented for consideration. A protoplasmic part which had previously been used to detect irradiation effect was caused to harbor changes brought about by radiation, carry them into a portion of non-irradiated protoplasm and there exert an influence which caused the untreated protoplasm to act as though it had been treated. This unique finding may be used in analyzing the action of radiation on the rate of cell division.

In the work with irradiated eggs, no information was obtained which indicated what parts of the eggs were affected by the radiation which, in turn, were responsible for the slowing of division rate. Modifications may have been produced in the membranes affecting permeability; they may have been produced in the cytoplasm causing alterations in viscosity, pH and other chemical organization; or they may have been produced in the nucleus, bringing about structural and chemical modifications. The situation, however, is different when sperm alone are irradiated.

When the normal egg is fertilized with normal sperm, the sperm penetrates the egg membranes, leaving its swimming organ at the surface. Thus, only the sperm nucleus and a small amount of cytoplasm actually enter the egg. The sperm nucleus moves forward through the egg cytoplasm to meet the egg nucleus and the two, so far as is known, share equally in the subsequent nuclear activity of the organism. In regard to the sperm cytoplasm which enters the egg, no important function has been attributed to it except one. It furnishes the functional centrosome for the first division figure, that of the egg not taking part. On entering the egg, the centrosome divides and moves to the poles of the egg, giving rise to the astral arrangement as it does so.

Thus certain points become clear. The irradiation effect which was transported to the egg by the sperm must have been carried by either the nucleus or centrosome or by both. From the fact that cleavage delay can be produced by irradiating sperm alone, it is clear that the delay does not result from alterations produced in the membranes nor in the greater bulk of cytoplasm such as that contained in the egg. Moreover, by considering the fact that the same irradiation effect is obtained when either sperm or eggs are irradiated, and the fact that the egg centrosome is not functional, the possibility of the centrosome harboring an irradiation effect which influences division rate is greatly diminished. The above results, therefore, may be cited as evidence indicating that the irradiation effect, as manifested by cleavage delay, is due to changes produced in the nucleus.

If this be true, the same evidence demonstrates in a clear manner that the nucleus exerts at least some control over the rate of cell division. X-rays have been used previously as a unique surgical tool in performing successful and effective operations on the nucleus, thereby bringing about changes in hereditary activity (Muller, 1928; 1933). The above experiments indicate that physiological activity may also be effectively modified by influencing the nucleus with this agent.

From a purely biological point of view, it is interesting to note that irradiation modification may be produced in a cell containing only half the normal number of chromosomes and that the effect becomes expressed in a cell containing the full number. Here, both an injured and a normal nucleus (haploid) are present in the same cell. It might be expected in such a case that the normal nucleus would exert a dominance over the abnormal one and thus prevent the irradiation injury from being expressed. This, on the contrary, is an example of an abnormal nucleus showing dominance over a normal one.

As yet nothing has been said in regard to the relative susceptibility of sperm and eggs to the radiation. The results, as presented, indicate that the effect is definitely greater when sperm are irradiated. If, as believed, the egg and sperm nuclei share equally in the nuclear functions of the organism, and if it is true that nuclear changes brought about by radiation are responsible for the radiation effect observed, it would be expected that equal effects would be produced on sperm and eggs by equal doses of radiation. The differences observed, however, are appreciable and tend to indicate a differential response on the part of the gametes. But there are reasons for believing that the differences observed are not real.

First, Heilbrunn and Young (1935) in a recent paper have reported work done on the same material and with the same source of radiation. In brief, they irradiated eggs in sea water in one case and in ovarian tissue in another and found noticably more cleavage delay when ovarian tissue was present during treatment. They suggest that this is evidence indicating the presence of *necrohormones*—an irradiation product which is produced in one tissue and which is diffused out and influences another. As a matter of convenience in our experiments, the eggs were irradiated in sea water and the sperm were irradiated in the presence of testicular material. Should necrohormones have been produced by this material, they would have caused the irradiation effect to be greater than it otherwise would have been. Second, the discrepency may be accounted for by differences in absorption in the two cases. The eggs settled to the bottom of the vessel during treatment and were protected by two to three millimeters of sea water, while at least part of the sperm were held near to the exposed surface by the testicular material. Since unfiltered radiation was used, two millimeters of sea water probably removed an appreciable amount of otherwise effective radiation. These factors, however, have not been investigated, thus making it impossible to discuss their significance at the present time.

Perhaps the most interesting phase of this investigation lies in the set of facts implied by the quantitative results. The reaction in its simplest form appears to be one of indirect dependence. That is, the effect observed (cleavage delay) is dependent on the amount of radiochemical change of a particular kind which is produced in the living system and which in turn is dependent on the amount of radiation administered. How the observed effect varies with respect to the quantity of radiation given is indicated by the shape of the curves obtained, but how the radiochemical change varies with respect to the dose of radiation applied and how the observed effect varies with respect to the amount of radiochemical change, is less obvious. The data, however, may be analyzed to obtain certain indicative information on these points.

Since it has been possible to produce the same irradiation effect by exposing either gamete, it is probable that the same radiochemical change (that which causes cleavage delay) is produced in the sperm and eggs indifferently. Through fertilization, then, two quantities of radiochemical change, known to produce definite amounts of cleavage delay, are brought together in the same system where their combined effects are expressed as one. This unique procedure, made possible only by the fact that cleavage delay may be produced by irradiating either gamete, offers an indirect method of investigating the quantitative aspects of the radiochemical change involved. For example, if the combined effect observed is equal to the sum of the two effects obtained independently, it follows that the observed effect is directly proportional to the amount of radiochemical change whatever it may be. Curve *D* in Fig. 1 shows the combined effects obtained by adding Curve *A* to Curve *B*.

The curve actually obtained when both elements were irradiated falls considerably below Curve *D*, indicating that the relation is not directly proportional. However, Curves *C* and *D* appear to be similar in shape, which indicates that the observed effect is equal to the combined calculated effect times some constant less than one. This has been found to be very nearly 0.72 at various points along the curves in the figure. Insofar as these results are trustworthy, this indicates that a linear relation exists between the radiochemical change produced and the biological effect observed. Since, however, the quantitative data now available are limited, it seems best not to carry the analysis farther at this time nor to emphasize the relationships observed. At this time it is sufficient to point out that a new method is available for investigating certain phases of the radiobiological reactions about which very little is known.

SUMMARY

1. Experiments have been carried out to investigate the influence of X-rays on the division rate of *Arbacia* eggs.

2. It was found, first, that the time required for the first cleavage was noticably prolonged when the eggs were irradiated before fertilization.

3. Second, it was found that the same effect could be produced by irradiating the sperm before they were allowed to fertilize normal eggs. Thus, an irradiation effect was carried by one gamete into another where the effect became expressed. The non-irradiated gamete was, therefore, caused to behave as though it had been irradiated.

4. Irradiation of both eggs and sperm indicated that the effect obtained was to some extent additive.

5. It was pointed out that only the nucleus and centrosome of the sperm appear to have an important influence on the egg and that the irradiation effect in such a case must have been carried by one or both of these. Reasons were given for believing that the centrosome was not important in this respect, thus indicating that the slowing of cell division observed was due to irradiation effects produced in the nucleus.

6. It appears, therefore, that the nuclei of the cells studied exert at least some control over the rate of cell division, and it is significant that such control is manifested when the damage is produced in a haploid nucleus and the effect is expressed in the presence of a normal haploid nucleus.

7. A method is presented for investigating quantitatively some of the more remote aspects of the radiobiological reactions.

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RECIPROCAL CHORIO-ALLANTOIC TRANSPLANTS OF EMBRYONIC DUCK AND CHICK KIDNEY TISSUE

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The results of reciprocal heteroplastic transplantations are not always the same. Such observations have appeared sporadically in the literature, particularly pertaining to grafting experiments between different species of amphibians. Similar observations on warm-blooded vertebrates are not as common. Loeb (1920) grafted kidney tissue between various forms. Some of these grafts were made reciprocally, insofar as host-donor relationships were concerned, between pigeon and guinea pig, and rabbit and guinea pig. More recently, Loeb and King (1935), and Loeb (1935*a* and 1935*b*) made a number of reciprocal transplantations which included such combinations as Gray Norway rats and mutant races, rat and mouse, rat and guinea pig, cat and rat, and rat and pigeon. Adult forms were used in these experiments, which involved transplants of thyroid, cartilage, ovary, and closely associated tissues. In general, the results showed that the grafts between the more closely related forms were more favorable than those between the distantly related forms. In the former there was some indication that a parallelism existed between the intensity of the reaction and the genetic relationship of the host and donor. In the latter, it was difficult to demonstrate this parallelism because necrosis appeared so rapidly in the transplanted tissue that it interfered with the more significant reactions which appear against living tissue. Under these conditions the results of the reciprocal transplants were, for the most part, similar, but some differences were noted.

Sandstrom (1932, 1934, and 1935), although not concerned with reciprocal transplants, reported a series of experiments designed to study the problem of heteroplastic grafting. His experiments were limited to that period in embryonic development when both the host and donor developed those properties responsible for the incompatibility displayed in adult tissue grafts. His results demonstrated that, in addition to the species specificity of the tissues, a number of interrelated factors must be considered in interpreting the results, e.g., the degree of differentiation of the donor tissue, the size of the transplant, the rate of incorporation of the transplant by the host, the capacity of the transplant to function in its new environment, and the possibility that the grafted tissue might become adapted to the host tissues.

The previous experiments (1932, 1934, and 1935) having been concerned with duck kidney tissue transplants to the chorio-allantoic membrane of the chick, the question arose as to whether grafts of the reverse host-donor relationship (i.e., metanephric tissue of the chick embryo grown on the chorio-allantoic membrane of the duck) would give similar results, and if not, whether a comparison of the reciprocal grafts would give any additional information pertaining to the general problem of heteroplastic transplantations.

A preliminary report on experiments, made with this thought in mind, has already been presented in abstract form (Sandstrom, 1933). During the interim additional transplants were made to fill gaps in the experimental series, and to corroborate earlier observations.

Material and Methods

The procedure used in these experiments was similar to that described by Sandstrom (1934). Briefly, it consisted of implanting a small bit of donor tissue on the chorio-allantoic membrane of the host where it was allowed to grow for the desired length of time, within the limits prescribed by this method of experimentation. In the present set of experiments, however, reciprocal transplants were made between duck and chick embryos, i.e., duck tissue was transplanted to the chick membrane, and chick tissue was transplanted to the duck membrane. The metanephros was again used as a source for donor material because it is an actively functional tissue (in contrast to cartilage, for example, which is considered passively functional), and, under proper incubation conditions, is known to differentiate in either homoplastic or heteroplastic chorio-allantoic grafts (Sandstrom, 1932). The donors were White Pekin duck embryos of 13, 16, and 24 days incubation, and single-comb White Leghorn chick embryos of 9, 13, and 19 days incubation. The hosts were White Pekin duck embryos and single-comb White Leghorn chick embryos of 14 and 9 days incubation, respectively.

Since the degree of differentiation is an important factor in determining the capacity of tissue to grow on the chorio-allantoic membrane, it was necessary, in order to make valid comparisons between the reciprocal transplants, to select hosts and donors of approximately the same stage in development. This is, of course, a problem in itself. The question immediately arose as to whether the chick, which hatches on the twenty-first day of incubation, is in the same stage of development at the time of hatching as is the duckling which hatches on the twenty-eighth day of incubation. Or is the latter more developed because of the additional week in the shell, and therefore equivalent to the one-week old chick? Either a gross, or histological study of the embryonic structures, such as is used in determining the degree of development in the earlier somite stages of avian embryology, for example, is of little value after visible differentiation has ceased. Since the metanephros was used again as donor tissue, and for the want of a better method in determining equivalent development, it was assumed that the changes occurring in the physico-chemical nature of the epithelial cells of the proximal convoluted tubules, as indicated by the manner in which they stored vital stains, would approximate the desired criterion. Hurd (1928) showed that trypan blue was stored in the form of bright granules in the metanephros of the chick embryo of 19 days incubation. Using the same kind of dye, Sandstrom (1935) determined that it was stored as well-defined masses in the kidney of the duck embryo of 24 days incubation. Assuming that these stages, therefore, are of equivalent development, from the ratio thus established, it was inferred that the chick and duck were approximately equivalent at the time of hatching. On this basis, insofar as it was practical, the ages of the hosts and donors used in the reciprocal transplantations of these experiments were selected.

The grafts were removed at 24-hour intervals after an initial period of 48 hours. They were fixed in Zenker's formal, sectioned at 6 and 8 micra, and stained with methylene blue-eosin. The present report is based on the study of 233 grafts.

Comparison of Grafts

Series I—Chick and Duck Donors of 9 and 13 Days Incubation, Respectively

At the time of transplantation, the metanephric tissue of the donors, whether chick or duck, possessed scattered tubules, and sometimes occasional renal corpuscles. It was still able to continue its differentiation when implanted on the chorio-allantoic membrane of the host, for considerable metanephrogenous tissue was still present. The actual condition of the transplanted tissue, however, varied according to the length of time the grafts were allowed to grow.

A comparison of the grafts of metanephric tissue of the chick embryo grown on the chorio-allantoic membrane of the duck for 2, 3, and 4 days, with those of the duck grown on the chick membrane for identical periods, showed similar conditions. All of the grafts were well incorporated. Differentiation had not progressed to any great extent in those removed on the second day; it appeared much as it did at the time of transplantation. In the grafts removed on the third and fourth days, the amount of metanephrogenous tissue was considerably reduced, and the transplants consisted largely of uriniferous tubules which, in some instances, could be differentiated into their various parts. But the tissue in the grafts removed up to this time was not all normal, particularly in those taken on the second day. Centrally, it appeared as though in the initial stages of necrosis. Instead of taking the methylene blue stain as did the more peripheral normal tissue, it stained with eosin only. The cytoplasm of the cells of the tubules contained vacuoles, and the nuclei were shrunken. This condition was undoubtedly due to the inability of the blood vessels to penetrate into the transplant rapidly enough to insure proper vascularization. Accompanying this apparently necrotic condition were numerous small lymphocytes. By the third and fourth days there was considerably more normal, and less necrotic, tissue. At the same time there occurred a proportional reduction in the number of small lymphocytes. They were usually limited to the small areas of tissue in which necrosis had definitely set in.

Upon comparing the reciprocal grafts removed on the fifth and sixth days, one prominent difference was noted which was not apparent in any of the transplants removed earlier. The incorporation of the metanephric tissue of the chick into the chorio-allantoic membrane of the duck was less complete than that of the duck into the chorio-allantoic membrane of the chick. The failure of the chick tissue to become completely incorporated manifested itself in two ways. In some instances, the grafts were taken into the host membrane, but only a small part of the surface of the transplant came in contact with the host tissue, and the region where the loose connective tissue and blood vessels of the chorio-allantoic membrane could enter was therefore limited considerably. Usually this contact was confined to one side. The remaining surface of the graft was separated from the host tissue by a space which was occasionally filled with a colloid-like substance. The tissue was normal.

In other instances, the grafted tissue was not completely incorporated. The impression gained from an examination of the sectioned material was that the chick tissue, although at first fully incorporated into the duck membrane, was being forcibly ejected. The inner side of the transplant was in contact with the host tissue, and the loose connective tissue and blood vessels of the host membrane entered the graft in this region only. The inner part, therefore, was, in a sense, incorporated, and was normal in all respects. But in passing toward the outer part, the tissue became more and more necrotic. The tubules were crowded, and their shapes altered considerably. It was still possible, nevertheless, to detect that some differentiation had taken place on the membrane. The outer part of the graft was definitely necrotic. It was not covered by the chorionic epithelium, the loose edges of which were found about the periphery, and the dead tissue was sloughing into the space between the chorionic surface and the inner shell membrane.

It is to be understood, however, that incomplete incorporation, such as described above, was not universal among the grafts of chick tissue on the duck membrane. Other grafts, in the minority to be sure, were incorporated fully as well as the duck-on-chick transplants. Regardless of the host-donor relationship, those grafts that were sufficiently incorporated to insure proper vascularization to all regions showed a high degree of differentiation with all parts of the typical kidney present, and little necrosis. Lymphocytes were not common, even in the grafts containing necrotic tissue.

There were more notable differences between the reciprocal grafts that were removed from the seventh to the tenth days. Although most of the tissue in the duck transplants grown on the chick membrane was normal, conditions were quite variable in the grafts of the reverse host-donor relationship. A few small grafts contained normal chick tissue, others showed varying amounts of necrotic tissue, and still others were in the process of sloughing in the manner already described. In some cases the transplanted chick tissue had completely sloughed from the host membrane, and the only evidence of a previous graft was an increased amount of connective tissue in the general region. In addition, there was another striking difference between the two types of transplants. In the majority of grafts of chick tissue removed from the duck membrane during this period, intense infiltrations of granulocytic leucocytes and many small lymphocytes made their appearance. Generally granulocytes are not uncommon in metanephric tissue grafts, but they are usually present in small numbers only. They are normally formed from the interstitial connective tissue of the developing metanephros, and their existence in the transplant is to be expected. But in the chick grafts removed from the duck membrane on, or after, the seventh day, they manifested themselves in a manner not found in any of the duck-on-chick grafts. They were so numerous that the transplant, in some instances, was completely obscured. They were also found in the connective tissue of the chorio-allantoic membrane immediately surrounding the graft. Wherever the granulocytes were sufficiently scattered so that the transplant could be studied adequately, its tissue was found completely incorporated and, for the most part, histologically normal. Some necrotic tissue, however, was present. The intensity of the granulocytic infiltrations could not be correlated with the amount of necrosis, for grafts which appeared almost completely normal were as intensely infiltrated as those that contained large necrotic areas.

A study of the chorio-allantois immediately around the grafted tissue revealed the source of the great numbers of granulocytes. It was quite obvious that the presence of the foreign tissue had stimulated the formation of granulopoietic centers, much in the same manner that homoplastic chorio-allantoic grafts of adult chicken spleen stimulated the formation of centers of granulopoiesis as described by Danchakoff (1918). In fact, many of the stages in the differentiation of the granulocytes, as described by her, were found. The loose connective-tissue cells had enlarged to form relatively large amœboid cells—the hemoblasts. Various stages could be selected to form a sequence of transformations leading from the hemoblast to the granuloblast. The final stage in this selected line of differentiation, the granulocyte, was very common. All of these stages were limited, for the most part, to circumscribed areas. These loci were located at various distances from the grafted tissue. In some cases they were directly adjacent to the transplant so that the various stages in the differentiation of the granulocyte were within the grafted tissue. More often, the loci, while in the immediate vicinity, were slightly removed from the graft, and in these cases, only fully differentiated granulocytes were found in the transplant.

Small lymphocytes, as well as granulocytes, were present, both in the grafted tissue and surrounding host membrane. They were not as common as the granulocytic leucocytes, and were somewhat larger than the small lymphocytes found in the necrotic-appearing areas of grafts removed 2 days after implantation. No evidence was found which would indicate the loci of their formation as coincident with the granulopoietic centers, although small lymphocytes were present in large numbers in these centers. Unlike the results of Danchakoff (*loc. cit.*), no convincing sequence of cell types could be selected that would suggest their transformation from the hemoblasts.

Series II—Chick and Duck Donors of 13 and 16 Days Incubation, Respectively

The reciprocal transplants of this series showed decided differences, although at the time of transplantation, the metanephroi of both donors had reached a comparable degree of histological differentiation. The metanephric tissue of the duck continued its growth and development on the chick membrane in the manner reported on previous occasions

(Sandstrom, 1932 and 1934). Considered briefly, the condition of the transplant varied according to the length of time the graft was allowed to remain on the host membrane. There was an initial period of 2 or 3 days when the final stages of incorporation were in progress, after which the implant was completely taken into the chorio-allantoic membrane and covered by the chorionic epithelium. During this period, the peripheral tissue was apparently normal, whereas the central part showed the early stages of necrosis, both as to its structural appearance and staining reactions. Numerous small lymphocytes were present in, and about, the necrotic area. Grafts removed subsequently, e.g., from the fourth to the tenth days, showed progressively less necrotic, and more normal tissue, as the length of time they grew on the membrane increased, so that ultimately their tissue, for the most part, was comparable to that of a normally developed metanephros of equivalent age. But some necrosis was present even in the grafts removed during the later intervals, the extent varying from small areas to complete grafts. The number of grafts containing necrotic tissue, however, was decidedly in the minority.

Upon comparison, the grafts of the reverse host-donor relationship, i.e., metanephric tissue of the chick grown on the chorio-allantoic membrane of the duck, showed conditions quite in contrast to the duck-on-chick grafts. A most striking difference was apparent in the process of incorporation, which, in most cases, was considerably retarded. Regardless of the length of time the grafts were allowed to remain on the duck membrane, the implanted tissue was rarely completely incorporated. It remained, more or less, in a stage comparable to that found in the duck-on-chick transplants removed from the host membrane within 48 hours after implantation. The epithelial cells of the membrane, undoubtedly stimulated in some manner by the presence of the implanted tissue, increased in number, forming epithelial projections which extended into the implant. Thus, that part of the metanephric tissue which was in immediate contact with the chorionic epithelium became incorporated first; it was "engulfed" by the host epithelium. Peripherally, the chorionic epithelium folded upward about the transplant, much as the somatopleure folds over the developing chick embryo in the formation of the amnion and chorion. Usually these folds extended upward only a short distance. Rarely did they cover the graft completely, and if so, not before the eighth day. In these cases, the inner part of the fold was sometimes found, much folded, in the mesoderm of the membrane, and not infrequently formed a hard, cornified mass.

Obviously, the tissue of the transplant was considerably affected by the retarded incorporation. That part which was "engulfed" by the epithelium was normal in all respects. It procured sufficient vascularization with little delay, and continued its development, so that the grafts removed from the eighth to the tenth days, for example, contained apparently normal, fully differentiated metanephric tissue. Necrosis appeared rapidly in the remaining unvascularized part, which was usually left outside the membrane, although occasionally it was completely covered by the chorionic epithelium. When covered, the dead tissue was always separated from the normal either by a space, or connective tissue.

Series III—Chick and Duck Donors of 19 and 24 Days Incubation, Respectively

The high degree of differentiation that the donor tissue, whether chick or duck, had attained at this stage of development, was reflected in the incapacity of the transplants to grow on the chorio-allantoic membrane. Regardless of the host-donor relationship, no great amount of normal tissue was found in the grafts. That which was present probably came from small scattered areas of metanephrogenous tissue that were still present at the time of transplantation, or represented tissue which was sometimes found to persist through the period that the grafts remain on the host membrane. Only one graft, a chick-on-duck, was completely normal.

One prominent difference was apparent between the reciprocal grafts of this series. The metanephric tissue of the chick was almost always completely incorporated into the duck membrane, even though it was seldom capable of continuing its growth. This complete incorporation of the implant was in striking contrast to the conditions found in similar grafts of the previous series (Series II), where incomplete incorporation was almost universal regardless of the stage of removal. It was also in striking contrast to the results obtained in the reciprocal grafts, i.e., duck-on-chick, of this series, which resembled, to a degree, the conditions prevalent in some of the chick-on-duck grafts of Series I. During the early period of incorporation, the implanted tissue, completely taken into the chick membrane, consisted of the characteristic peripheral area of normal tissue and the relatively larger central area of apparently necrotic tissue. Numerous small lymphocytes were aggregated in and about the central part. From the fourth day on, however, much of the grafted tissue was found in the process of sloughing. Not infrequently, it had sloughed from the membrane completely, and the area once occupied by the duck tissue was filled with connective tissue.

DISCUSSION

The foregoing comparison of the embryonic duck and chick transplants demonstrated that a definite reciprocal relation existed between the host and donor species only during early embryonic development. During this period the metanephrogenous tissue of both the duck and chick were equally well incorporated into the chorio-allantoic membrane of their respective hosts. As development proceeded, however, this reciprocal relation was, to a considerable extent, lost. Pronounced differences appeared in the transplants. For example, the implanted metanephric tissue of the 14-day chick embryo was incorporated less completely, and more slowly, than the equivalently developed tissue of the 19-day duck embryo was into the chick membrane. This retarded incorporation is demonstrated, not only in many of the grafts of Series I (donors of 9 days incubation) that were removed from the host membrane on, and after, the fifth day, but somewhat more decisively in most of the grafts of Series II (donors of 13 days incubation), irrespective of the time of removal.

Other differences were noted. In Series I, completely incorporated chick tissue, after having grown in the new environment from 7 to 10 days, often stimulated the formation of leucopoietic centers in the neighboring regions of the duck membrane. No stimulation of this kind resulted from the transplantation of duck tissue to the chick. Furthermore, in Series III, the implants of fully differentiated, functional metanephric tissue from both the chick and duck (ages of 19 and 24 days incubation, respectively) were completely incorporated, with no obvious delay, into the membranes of their respective hosts. In this group, however, it was the transplanted chick tissue that grew, or at least persisted, in its new environment, whereas the grafted duck tissue, after having grown for a few days, frequently sloughed from the membrane without creating any apparent disturbance in the neighboring host tissue. And finally, closely correlated with the retarded rate of incorporation, considerably more necrotic tissue was found, in general, in the grafts of chick tissue than in those of duck tissue.

Undoubtedly, the reactions described in the foregoing paragraph were associated with the development of species specificity in the donor tissues. Its appearance in the metanephric tissue of the chick obviously occurred on the fourteenth day of incubation, the time when the incorporation of the chick tissue was first retarded. But as for the appearance of species specificity in the duck tissue, no actual evidence is available from these experiments. Assuming that the method used in determining equivalent development is essentially correct, it should be present at approximately the nineteenth day of incubation. As a matter of fact, this conclusion checks with that of Sandstrom (1934) who, from a study of heteroplastic and homoplastic chorio-allantoic grafts of duck kidney tissue, experimentally demonstrated its presence at this time.

Although it is essential that the transplanted metanephric tissue should function in order to continue its growth on the chorio-allantoic membrane, as was shown by Sandstrom (1934 and 1935), apparently its incapacity to do so does not elicit a reaction from the host. At least this was true for the duck-on-chick transplants. More than likely, therefore, the lack of function in the chick-on-duck transplants cannot be considered an important factor in causing the reactions found in these grafts. That the metanephros of the chick has developed function by the fourteenth day of incubation is suggested by the results of Gersh (personal communication). By a refined technique, he has been able to demonstrate activity in the tubules of the chick metanephros as soon as they are structurally differentiated, i.e., starting the eleventh day. According to his observations, however, only a very few tubules are sufficiently differentiated at this time, and the number increases but slowly, making it highly improbable that the incapacity to function played an important part in instigating the reaction obtained.

Since the species specificity of the transplanted tissue was the primary factor responsible for the reactions between graft and host, the differences in these reactions, as found upon comparison of the reciprocal grafts, must be ascribable to differences in the capacity of this factor in the donor tissues to instigate reactions in the membranes of their respective hosts. The more determined reactions obtained from the chick implants, as compared with the duck, suggest that either the species specificity factor of the former is the more potent of the two, or the chorio-allantoic membrane of the duck is the more reactive. In either case, it would be the donor that provoked the reaction, the character of which would necessarily have to be a function of the host. The reactions obtained in the reciprocal grafts, therefore, differed. These results are well in accord with the statement made by Loeb (1930, p. 572) that "if the relation between host and donor is reversed, the results of transplantation, as manifested in the intensity of the reaction against the transplant, do not need to be identical; no simple reciprocity therefore exists. This can be readily understood, if we consider that it is the host which reacts against the transplant, and not the transplant which reacts against the host, or at least that, under ordinary circumstances, we have no means of determining the reaction on the part of the transplant against the host."

Incidentally, it is difficult to understand how the donor tissue, without reacting against the host, can provoke a reaction in the host, as implied in the statement by Loeb, quoted in the previous paragraph. As a matter of fact, an analysis of the process of incorporation, as it occurred in the chorio-allantoic grafts of chick tissue on the duck, suggests that the donor does react against the host. Although chorio-allantoic grafting as a method for study of heteroplastic transplantation may possess certain shortcomings, it has the advantage over other methods in that the incorporation of the implant can be used as an index of reactivity. The implant is carefully placed on the surface of the chorionic epithelium, so that the epithelial tissue is involved before the foreign transplant finally becomes lodged in the connective tissue of the membrane. No actual mechanical injury, therefore, is incurred by the host. This method is in contrast to that of subcutaneous transplantation, for example, as practiced with adult animals, in which the implant is placed directly into the subcutaneous connective tissue, thereby mechanically assisting the process of incorporation. In addition, since mechanical injuries to the recipient's tissues are unavoidable results of this procedure, the incorporation of the implanted tissue cannot be used in the study of either host or donor reactions. In the chorio-allantoic grafts under consideration, therefore, the process by which the implant was incorporated assumed considerable importance. The only connection between the host and donor tissues at the time of implantation was a physical contact between the surfaces of the implant and chorionic epithelium, and, it will be recalled from the description of the results, that the epithelium subsequently "engulfed" portions of the implanted tissue. Under these conditions, the rate of incorporation was determined by two opposing factors—an apparently natural tendency for the epithelium to react favorably toward the implant, as indicated by the manner in which it "engulfed" parts of the implanted tissue, and the presence of the species specificity factor in the donor tissue which inhibited the "engulfing" activity of the host. The amount of tissue incorporated was apparently dependent on the relative effectiveness of these two factors. In any event, the analysis has indicated that some of the responsibility for the retarded incorporation rests with the reaction of the transplant against the host.

It has been emphasized that the species specificity factor of the donor tissue was of primary importance in causing the reactions in the transplants; therefore further consideration must be given to the seemingly contradictory results obtained in the grafts of Series III, in which fully differentiated, species specific tissue was not only readily incorporated, but even failed to call forth a reaction from the host. From the results of the chick-on-duck transplants it was evident that, insofar as its capacity for eliciting a host reaction is concerned, the developing donor tissue passed through three stages: during the first, which obtained previous to the development of species specificity, the host and donor tissues were compatible; the second obtained during the development of species-specific properties which caused a definite reaction; and the third obtained subsequent to the appearance of species specificity when no reaction occurred. Owing to the milder response, these stages, although present, were not very apparent in the duck-on-chick grafts.

Such an unusual sequence is not unparalleled among grafting experiments, for an analogous situation was found existent in some of the results mentioned by Loeb (1930) in a review of his theory of organismal differentials. This author, referring particularly to the intensity of the reaction which appeared against his transplants of non-resistant tissues (e.g., thyroid, made between variously related adults), as indicated by the lymphocytic infiltrations, stated that few, or no, lymphocytes were found in autoplastic transplants. According to Loeb's conception, the lymphocytic reaction is a response toward a toxin produced by the transplant. Inasmuch as host and donor were the same individual, and had identical individuality differentials (chemical characteristics), no toxic substance was produced to attract the lymphocytes. In homoplastic transplants, although the respective individuality differentials of the host and donor differed, this difference was not great enough to interfere with the metabolic activity of the transplant. Sufficient toxin was produced, therefore, to call forth a decided infiltration of lymphocytes. In heteroplastic transplants, the individuality differentials of the host and donor were so different that the body fluids of the former, acting directly on the latter, caused degenerative changes therein. These changes interfered so extensively with the metabolism of the implant that only a slight amount of toxin was formed. As a result, the intensity of the lymphocytic reaction was less pronounced, and appeared more slowly, than that which appeared against the homoplastic transplants.

Insofar as the intensity of the reaction is concerned, therefore, the sequence obtained in the chorio-allantoic grafts of developing metanephric tissue not only seems to recapitulate the conditions described in the preceding paragraph, but the explanation postulated by Loeb, in consideration of his results, may also be applicable. The conditions found in the chick-on-duck grafts of the first stage are comparable to those found in the autoplastic transplants; no reactions occurred. In the grafts of the second stage, however, the intensity of the reactions resembled that of the lymphocytes in the homoplastic transplants. The individuality differential of the donor had developed sufficiently to excite a reaction without interfering appreciably with its capacity to live in the new environment. Furthermore, the implanted tissue, because it was still undifferentiated enough to be plastic, could be handled, etc., during the operative procedures without causing considerable degenerative changes. Thus this metabolically active tissue, perhaps through the agency of a toxin, affected the host tissues in a decisive manner. In the final stage of the sequence, e.g., grafts of Series III, true heteroplastic conditions were being approximated; the development of the individuality differential (species specificity factor) had reached completion. Even though the differentials of the host and donor resembled one another less than in the previous stage, the host was unresponsive toward the implant during the limited time it remained on the membrane. As in the case of heteroplastic transplants of Loeb, the intensity of the reaction was undoubtedly diminished because of degenerative changes that occurred in the implant. To what extent the body fluids present in the chorio-allantoic membrane of the unhatched host were capable of causing these changes is doubtful. It is more than likely that the changes were caused by injuries incurred during the operative procedure, since the plasticity of the tissue had decreased considerably. In addition, vascularization, so essential to a differentiated, functional tissue, was necessarily retarded because the process of incorporation, as it occurs in chorio-allantoic transplants, at best, is relatively slow. Under such conditions the implant could not have consisted of an actively metabolizing tissue, and thus lacked the essential prerequisite for calling forth a reaction.

SUMMARY

1. Metanephric tissue from duck and chick embryos that were approximately equivalent in development was transplanted to the chorio-allantoic membrane of the chick and duck respectively. The hosts, also, were in comparable stages of development.

2. A reciprocal relation was found to exist during the early embryonic stages only. As development progressed this relation was lost, to a large extent; prominent differences occurred in the two types of transplants. Whereas relatively slight or no reactions were observed in the duck-on-chick grafts, intense reactions appeared in the grafts of the reverse host-donor relationship.

3. The reactions appearing in the grafts were associated with the development of the species specificity factor in the donor tissue.

Its appearance in the chick tissue occurred at about the fourteenth day of incubation.

4. The reactions which appeared as a result of the development of the species specificity factor were: (1) a decided retardation of the rate of incorporation of the implanted tissue; (2) possible expulsion of the transplant from the membrane; and (3) the stimulation of leucopoietic centers in the connective tissue of the surrounding host membrane. These reactions, if present in the duck-on-chick grafts, were scarcely observable because of their mildness.

5. It was demonstrated by these experiments that, although the host reacted against the transplant, which obviously instigated the reaction, it was also possible, as was shown by an analysis of the process of incorporation, for the transplant to react against the host.

6. Seemingly contradictory results were obtained in the grafts of fully differentiated, species specific tissue, in that the implants were readily incorporated into the host membrane, without provoking a reaction. Although no evidence was offered, an explanation of these unusual results was suggested which was based on the theory of Loeb (1930) that the reaction against a transplant is dependent, to a large measure, on the active metabolism of the transplanted tissue.

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EXPERIMENTAL STUDIES ON THE NUPTIAL PADS OF MALE TRITURUS VIRIDESCENS

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During the breeding season the adult male *Triturus viridescens* develops heavy black nuptial pads on the inner aspect of the femoral area and toe tips. There is also a marked hypertrophy of the tail fin, swelling of the cloaca, and development of the hedonic glands (Hilton, 1902). Adams (1932) reported the experimental production of leg-pad development, tail-fin response as well as ovulation and moulting following the administration of anterior pituitary extract (*Phyone*) in this species. Increase in tail-fin size was not interpreted as a secondary sexual character under the influence of the gonad since it can be produced with *Phyone* injections in castrates. These results have been confirmed by Dawson and Jimenez (1933) who, in addition, demonstrated that the hedonic glands responded in the manner characteristic of secondary sexual characters. Similar logic has been applied by Adams (1931) in a study of the effect of intramuscular injections of anuran pars anterior in immature *T. cristatus*. Here dorsoventral expansion of the tail and head enlargement were produced in both normal and castrated animals.

MATERIALS AND METHODS

The femoral pad is of value in experimental studies as an indicator of the hypophysis-gonad-reproductive tract inter-relationship. The writer was attracted to this area as a useful indicator for studies on the

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Explanation of Figures

1. Ventral view of an animal in which its left femoral pad has been exchanged with the skin of the pectoral area (autoplastic series) showing hypertrophy and pigmentation of normal and transplanted tissue following six injections of pituitary extract. Photograph taken three hundred and thirty-five days after graft was made.
2. Pectoral area of same animal at higher magnification.
3. Ventral view of a newt in which a femoral pad was grafted from another animal of the same species to the pectoral region (homoioplastic series). Typical response of secondary sexual characters was obtained for the normal host tissue following seven doses of pituitary extract. The homoioplastic transplant shows no corrugation or pigmentation. Photograph taken sixty-six days after transplantation.
4. Pectoral area of same animal at higher magnification.

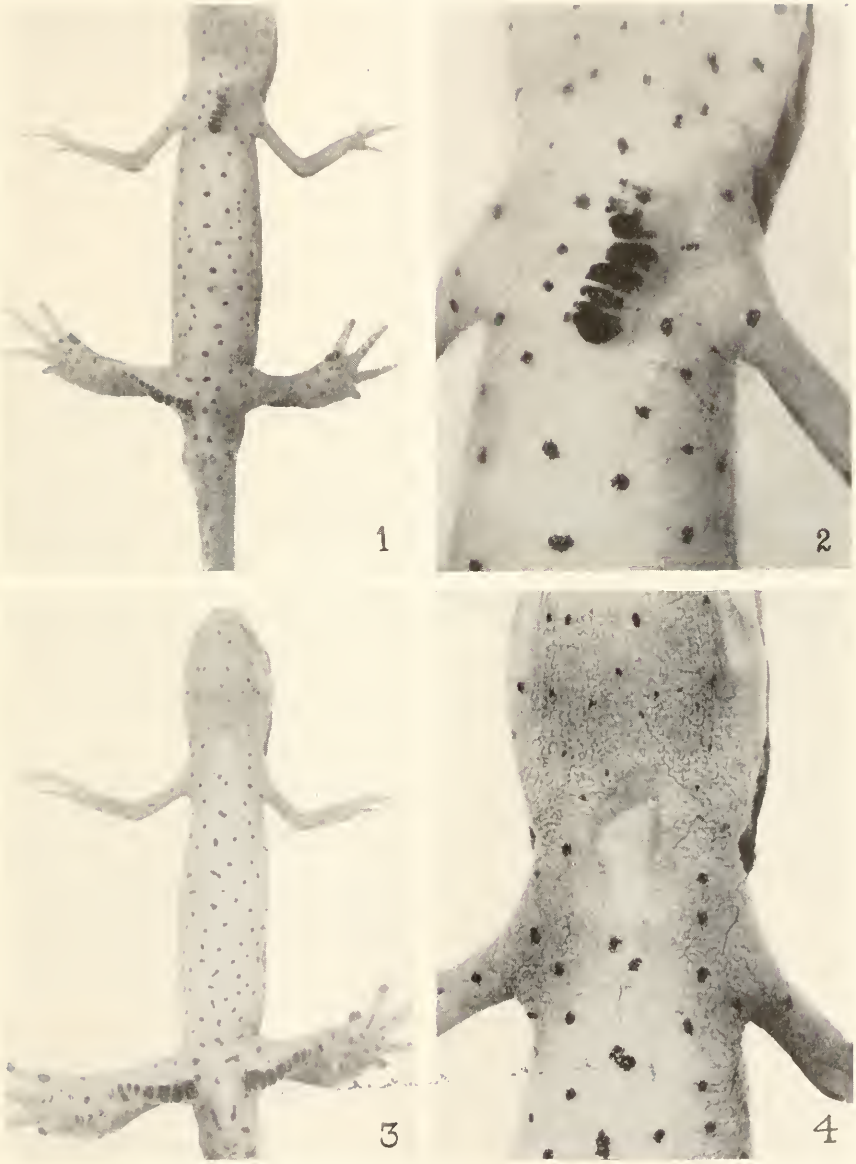


Plate I

persistence of skin transplants. The general technique employed in this work consisted in cutting out the entire femoral area from one limb of an animal under chloretone anaesthesia and transplanting it to the midventral line between the forelimbs. Upon stimulation with intraperitoneal implants of frog anterior pituitary or an extract of whole sheep gland it was possible to determine whether the grafted tissue had persisted by referring to the pad of the uninjured leg. Only sexually mature aquatic male newts were used. All experimental animals were kept in aquaria at room temperature.

RESULTS

Autoplastic transplants were consistently found to persist unchanged in eight animals after more than three hundred days. Figures 1 and 2 illustrate the results obtained in a typical case three hundred and thirty-five days after autotransplantation. Stimulation of secondary sexual characters was brought about by means of six doses of pituitary extract (whole sheep gland, Parke Davis and Company). Each dose consisted of an intraperitoneal injection of 0.2 cc. of a 1–5 aqueous solution. Injections were made every second day. Adequate response is evidenced (Fig. 1) by the development of hypertrophy and pigmentation of toe pads and the normal (right) femoral area. The left femoral pad is seen in the pectoral position and this appears typical (Fig. 2).

In eight cases of homoiotransplantation the femoral pad was never found to perist unchanged for more than sixty days. Progressive removal or rearrangement of grafted tissue was evidenced by the failure of characteristic hypertrophy of the transplanted pad tissue following pituitary stimulation. Figures 3 and 4 illustrate a typical

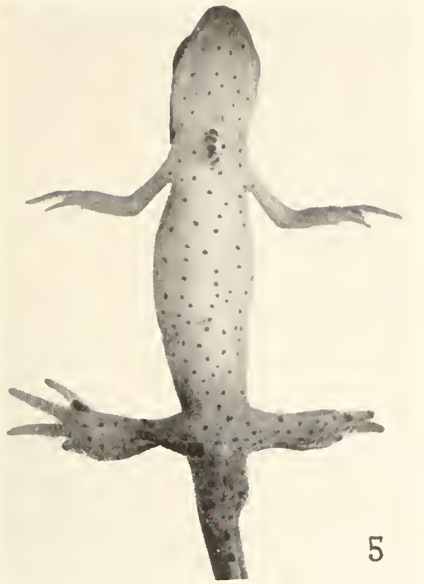
Explanation of Figures

5. Ventral view of an animal one hundred and fifty days after amputation of left limb. An autoplastic transplant of the femoral pad was made at the time of amputation. Graft shows a reaction similar to the normal right leg which serves as control to the action of seven doses of pituitary extract.

6. Ventral view of an animal seventy days after amputation of the right limb. An homoioplastic transplant of femoral pad tissue was made at the time of amputation. The normal left leg shows characteristic reaction to seven doses of pituitary extract but the homoiotransplant seems almost entirely resorbed.

7. View of the ventral aspect of the pelvic area and left limb regeneration seventy days after amputation. The first toe shows definite development of pigmentation in response to seven injections of pituitary extract.

8. Ventral aspect of regenerated limb three hundred days after amputation. The form of the toes is not typical. Pigment pads have developed on all toes and there is evidence of scattered masses of pad tissue along the post-axial margin near the base of the limb.



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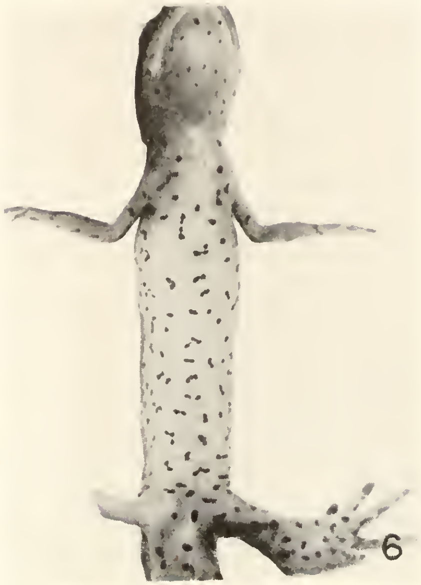

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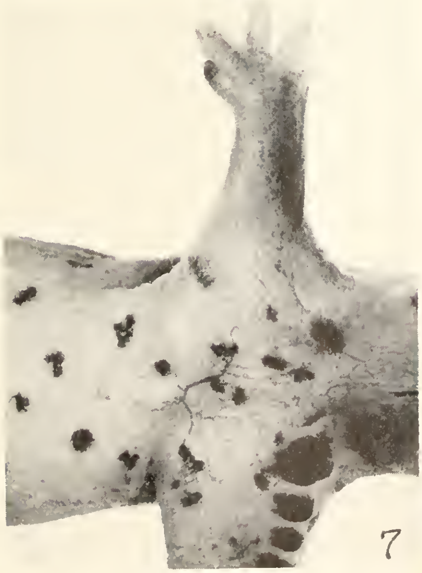

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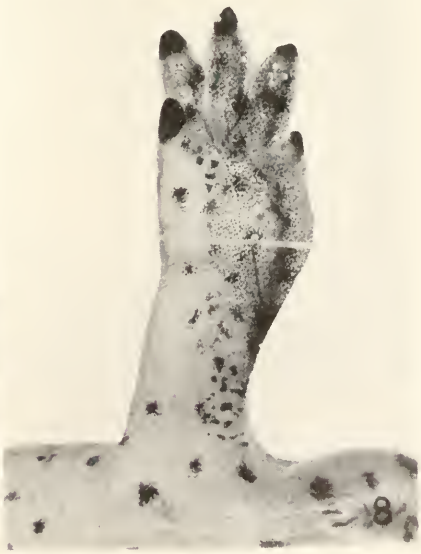

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Plate II

homoiotransplant in which the secondary sexual areas of the host were undisturbed and have given characteristic reactions following seven doses of pituitary extract. Photographic study at higher magnification (Fig. 4) of the pectoral graft shows complete absence of pigmented tissue.

Both auto- and homoiotransplantations have been performed on a series of animals in which limb regeneration was being studied. Confirmation of the results reported above was obtained (Figs. 5 and 6), thus excluding the possibility that lack of persistence of homoiotransplants might be due to the presence of pad tissue in excess of the normal amount.

Femoral pads were never observed to regenerate following extirpation. Denuded femoral areas to which skin from the pectoral region was transplanted failed to organize nuptial pads after more than three hundred days (Fig. 1).

Following amputation, limbs were found to regenerate but in no case were they found to equal the normal limb length nor were the relative length and form of the toes typical. These results confirm those reported by Collins (1932).

During regeneration the nuptial tissue was found to appear first in the toe pads. A definite, pigmented area was observed on the first toe tip seventy days after amputation (Fig. 7). Toe pigmentation then proceeded serially in a post-axial direction. Definite pigmentation in the femoral area was first observed at the end of the three-hundredth day of limb regeneration (Fig. 8). It was not possible to determine whether this process extended proximo-distally or disto-proximally.

SUMMARY

The femoral pads of the newt, *Triturus viridescens*, respond to pituitary extract stimulation by characteristic hypertrophy and pigmentation. Autoplastic transplants of pads were found to persist intact after more than three hundred days while homoioplastic transplants lost the capacity to exhibit normal reactions after sixty days. This is not a function of the total amount of pad tissue present. Regeneration of the femoral pads in denuded areas was never observed. Regenerated limbs showed pigmentation of the first toe within seventy days. Evidence of the reorganization of the femoral pad was noted three hundred days after amputation.

It is a pleasure to acknowledge the helpful advice generously given by Dr. A. B. Dawson and Dr. H. W. Rand.

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OBSERVATIONS ON AN ENDAMŒBA PARASITIZING OPALINID CILIATES

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INTRODUCTION

Endamœba is well known as a parasite of Metazoa, but it had not been identified as a parasite of Protozoa until 1933, when a brief notice of this remarkable phenomenon was published by Stabler. The protozoans found infected with *Endamœba* were opalinid ciliates, living in the rectum of frogs and toads.

The first observers of endamœbæ within the opalinids either did not recognize them as parasites at all or identified them incorrectly. Thus, Metcalf (1923) described them as "secondary" and degenerating nuclei which he believed to be "abnormal" and "associated with parasitism." Carini (1933*a* and *b*) recognized them as parasites, but thought they represented a new genus which he called *Brumptina*, in honor of Professor Brumpt, who had seen but not identified the same objects in 1913. Since Stabler (1933) recognized these "secondary nuclei" and "*Brumptina*" as endamœbæ, the report has been confirmed by the recent observations of Carini and Reichenow (1935).

In the present contribution, we report further observations on the endamœbæ infecting opalinids from a number of different localities. These observations have been supplemented by independent examination of the original preparations of Metcalf and Carini.

MATERIAL AND METHODS

The material consists of the following opalinids parasitized by amœbæ: *Zelleriella hirsuta* in *Bufo cognatus cognatus* from Flagstaff, Arizona; *Z. opisthocarya* in *B. marinus* from Panama City, Panama; zelleriellas in *B. woodhousii* from Somerton, Arizona and from the vicinity of Tuba City, Arizona; and zelleriellas in *Pleurodema bibroni* from the vicinity of Coquimbo, Chile. A number of other species of anurans and their opalinids from different localities were also examined, but no endamœbæ were found in them.

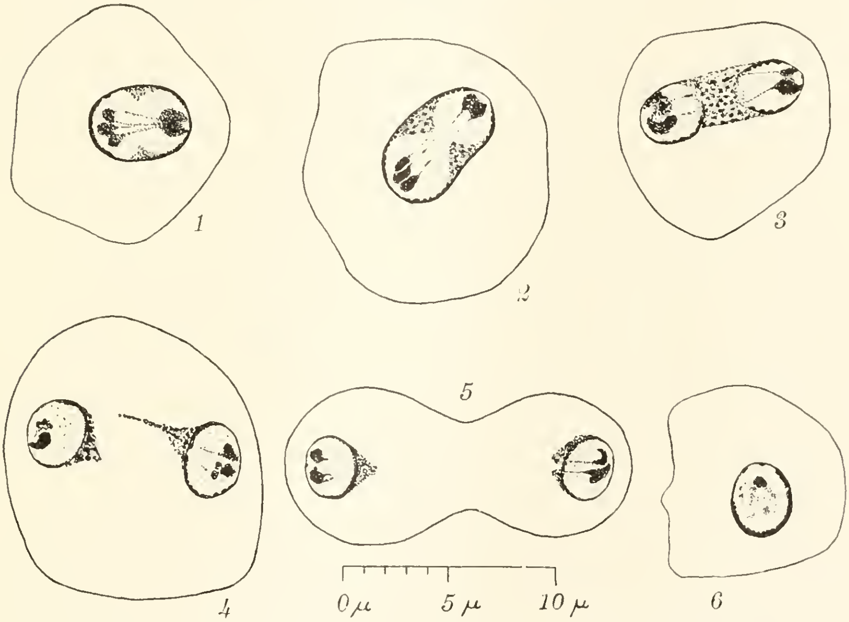
The ciliates containing the parasites were studied to some extent in 0.8 per cent NaCl and in dilute Lugol's iodine solution, but mainly on smears fixed in warm Schaudinn's fluid containing 5 per cent of glacial acetic acid and subsequently stained in Heidenhain's hæmatoxylin. Some of the destaining was carried out in iron alum, some in a saturated aqueous solution of picric acid.

All drawings were made with the aid of the camera lucida.

Original Observations

General Description

The organism found living in the cytoplasm of these opalinids is an *Endamœba*, with all the features characteristic of the genus. It greatly resembles *Endamœba ranarum* morphologically.



Figs. 1–6. Late division stages in the endamœbæ. For explanation see text.

Observed in the living condition, the amœbæ appear as small refractile bodies within the cytoplasm of the actively moving ciliates. Due to their small size, they could easily be overlooked, as the opalinids turn and dart about. In the iodine solution, however, they are easily discernible and their *Endamœba* characteristics readily noted. The amœbæ seem to have little or no effect on the ciliate. Not only do the ciliates move actively about, but they also undergo normal binary fission despite the presence of numbers of amœbæ in their cytoplasm (Fig. 22).

Although a good number of species of anurans and their opalinids from different localities have been examined, only certain species of anurans from some localities and a certain percentage of these latter individuals contain ciliates parasitized by the endamœbæ. Within an individual anuran host, if the opalinids are thus parasitized, a number of these ciliates may still be entirely free from the amœbæ, some harboring a single amœba, others a number of them (Fig. 23) and still others may have their cytoplasm filled with well over a hundred of these parasites (Fig. 24).

The amœbæ are often seen lying in pockets in the cytoplasm of the ciliates and occasionally two or more amœbæ are found in the same pocket (Fig. 10). The space between the amœbæ and the cytoplasm may represent, partially at least, a shrinkage space resulting from fixation and dehydration. A number of amœbæ have been found exerting such pressure on the nuclear membranes of the opalinids that the membranes were markedly indented (Fig. 8). No amœba has ever been observed within a nucleus, however. Usually all the amœbæ in any one ciliate are in the same stage of development, i.e. all cysts or all trophozoites (Figs. 22, 23, and 24).

DESCRIPTION OF PLATES

Drawings all made with the aid of the camera lucida from material stained in Heidenhain's hæmatoxylin and destained in iron alum except where noted.

Explanation of Plate I

Figs. 7–18 are of the *Endamœba* living in the opalinids.

Figs. 19–21 are of *Endamœba ranarum*.

Fig. 7. Trophozoite in the periphery of *Zelleriella* showing ingested endospherules.

Fig. 8. An amœba indenting the nuclear membrane of the ciliate.

Fig. 9. Trophozoite, the nucleus of which shows an abundance of stainable material about the karyosome. Destained in a saturated aqueous solution of picric acid.

Fig. 10. Two amœbæ in the same pocket.

Fig. 11. Two nuclei in the same amœba.

Fig. 12. An amœba outside the opalinids. Note absence of food bodies and the abundance of stainable material in the center of the nucleus. Destained in picric acid.

Fig. 13. Amœba containing the *Sphærita*-like organisms. Destained in picric acid.

Fig. 14. Trophozoite showing the typical *Endamœba* features.

Fig. 15. Typical uninucleate cyst within a *Zelleriella*.

Fig. 16. A very small trophozoite. Destained in picric acid.

Fig. 17. Quadrinucleate cyst outside the ciliates. Note the *E. coli*-like chromatoids.

Fig. 18. Quadrinucleate cyst outside the ciliates. Note the *E. histolytica*-like chromatoid. Destained in picric acid. Compare with Fig. 21.

Fig. 19. *E. ranarum* trophozoite. Note ingested bacteria.

Fig. 20. Uninucleate cyst of *E. ranarum*.

Fig. 21. Quadrinucleate cyst of *E. ranarum*. Compare with Fig. 18.

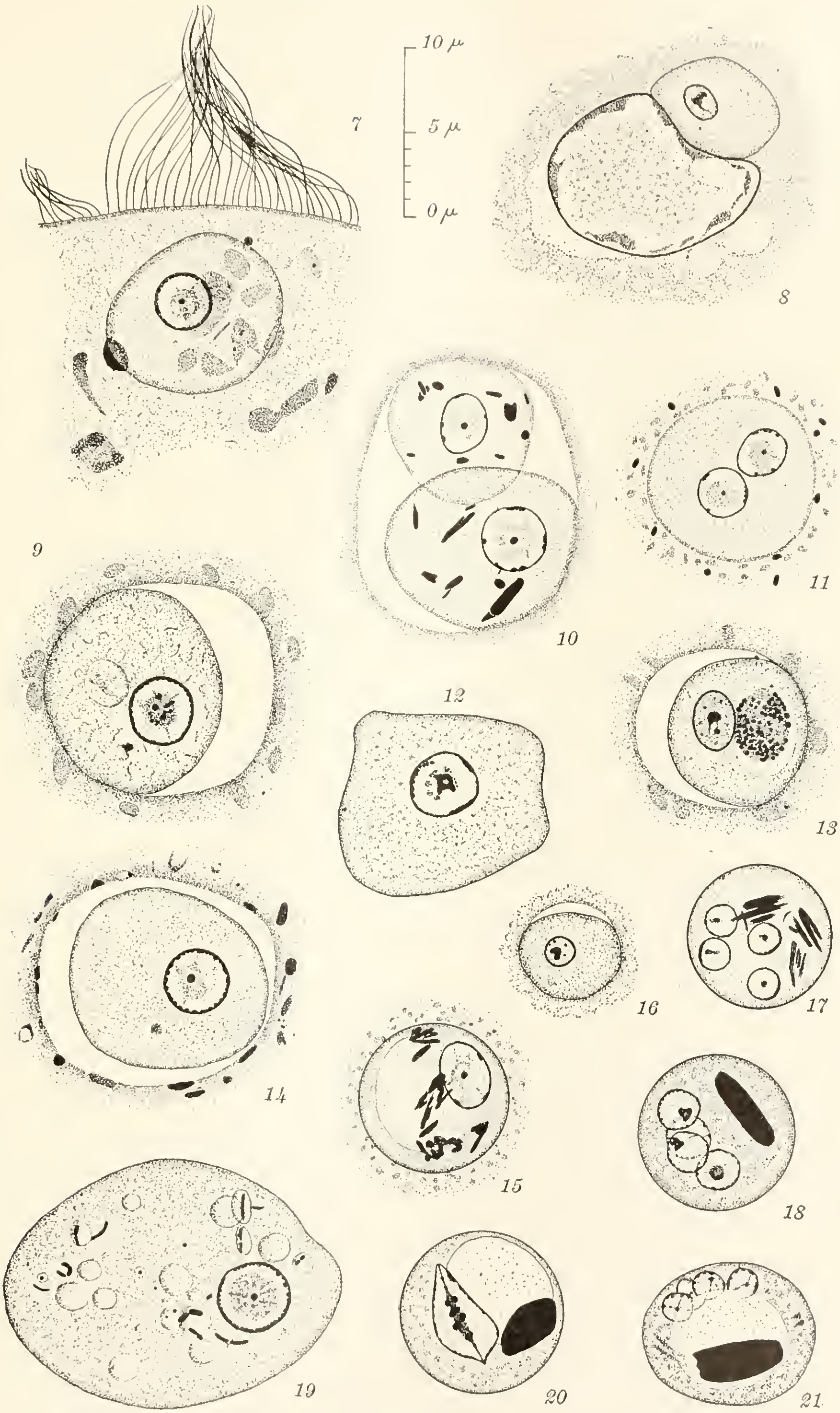

10 μ
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PLATE I

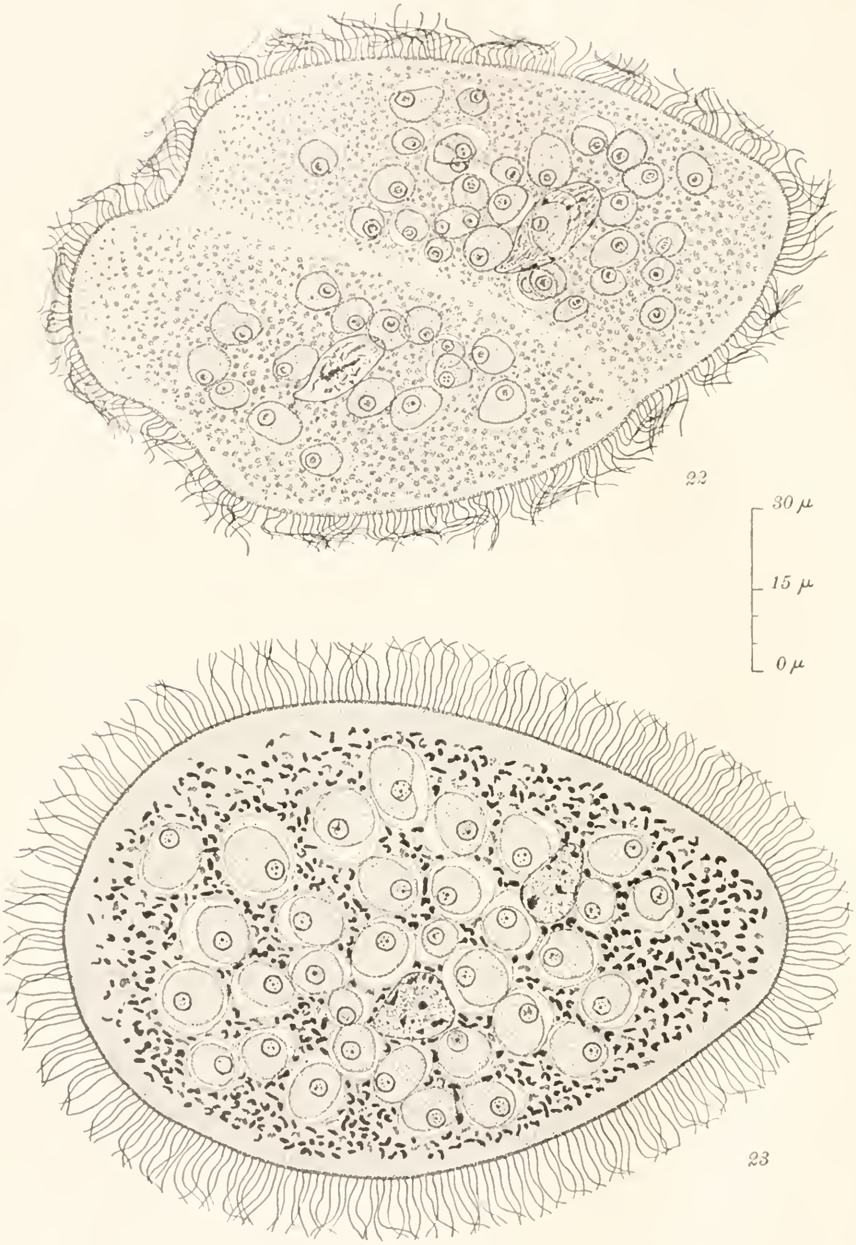


Plate II

Fig. 22. A *Zelleriella* in a late stage of binary fission, containing many endamœbæ. Cilia diagrammatic.

Fig. 23. A *Zelleriella* fairly well filled with the trophozoites of the *Endamœba*. Cilia diagrammatic.

Although there may be these hundreds of amœbæ within the ciliates, relatively few are found free in the lumen of the rectum of the anuran host (Fig. 12). These latter are thought to represent amœbæ that have either escaped from, or been freed by the disintegration of the ciliates.

Trophozoites

The amœba is a fairly small one; seventy-five nearly spherical trophozoites taken at random from a number of different hosts range in diameter from 5.3 μ–14.3 μ, averaging 8.0 μ. Some ciliates contain enormous numbers of very small amœbæ only, while others harbor the large forms.

The amœbæ present typical *Endamœba* nuclei. In well-fixed material there is usually a fairly even ring of peripheral chromatin at the nuclear membrane (Figs. 7, 9, and 14). At times, however, this ring is broken into masses and blobs (Figs. 10, 11, and 12). A karyosome, which frequently appears as a discrete granule surrounded by an area of more lightly staining material (Figs. 7, 10, 11, and 14), shows particularly well when the stain has been differentiated in iron alum. After picric acid destaining this karyosome is usually less prominent and the surrounding material more deeply stained (Figs. 9 and 12). Occasionally two nuclei are found within the same amœba (Fig. 11).

A number of dividing stages have been observed in the trophozoites. However, only the later stages of mitosis have been recognized, an anaphase being the first stage of which we are certain (Fig. 1). In these later mitotic stages, in the mid-region of the elongating nucleus, there appears to be the progression of a series of granules from the nuclear membrane inward (Fig. 1). These granules do not appear to contribute to the formation of the chromosomes. As the daughter nuclei separate, this area of granules becomes larger (Figs. 2 and 3) and is finally stretched out as a "tail" from each of the telophase nuclei (Figs. 4 and 5). These granules finally disappear entirely and the reorganizing nucleus is featured by the layer of heavier peripheral chromatin on the one side of the membrane and the decidedly scarcer chromatin on the other (Fig. 6). This distribution of peripheral chromatin is typical of all the late telophase nuclei. It never entirely disappears from the nuclear membrane during mitosis. The exact chromosome number could not be determined from our material.

The question as to the food habits of these amœbæ is a difficult one to answer. They apparently feed on the endospherules of the opalinids, since these are the only inclusions noted in the trophozoites (Fig. 7). The apparently identical amœbæ found outside the ciliates contain no solid food.

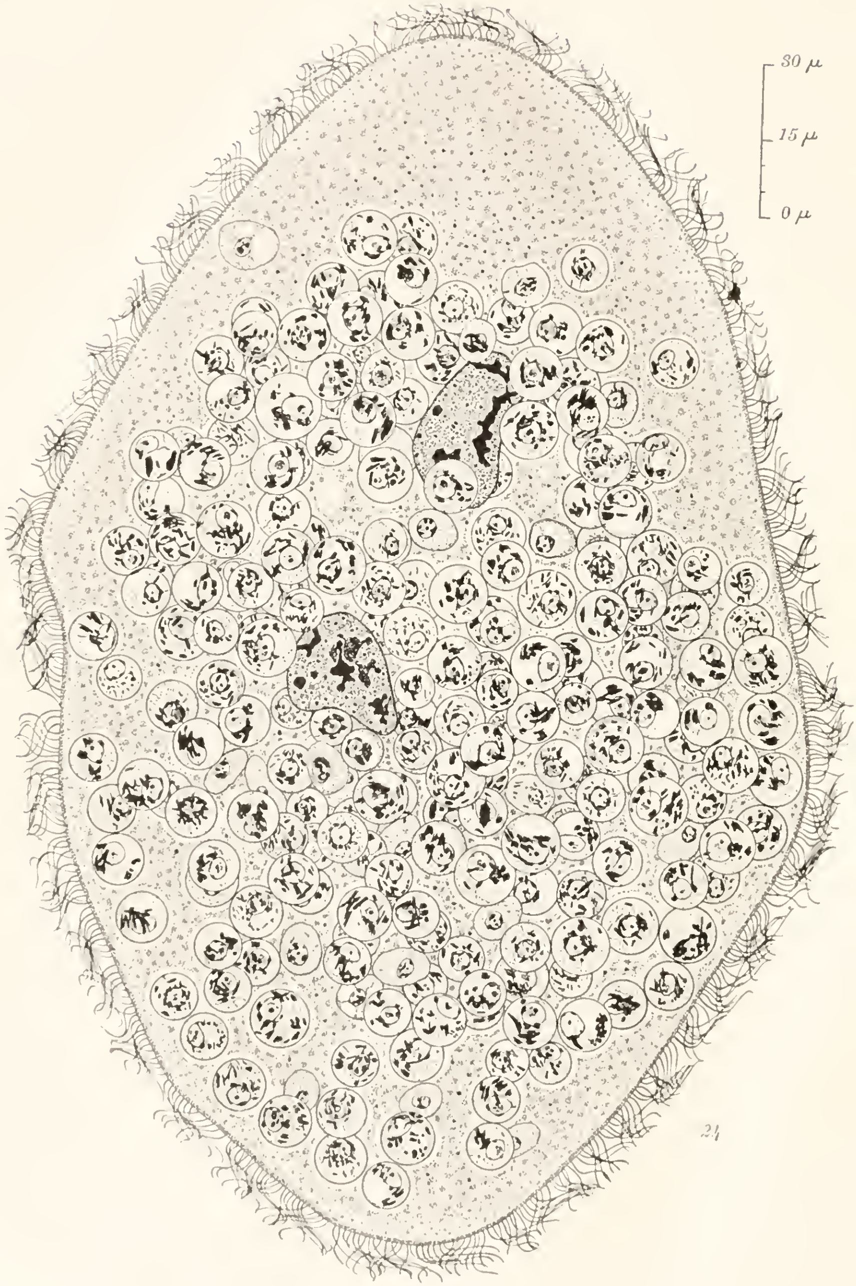


PLATE III

FIG. 24. *Zelleriella opisthocarya* containing over two hundred cysts of the *Endamœba*. Note uninucleate condition of the cysts, type of chromatoids, etc. Cilia diagrammatic.

Cysts

The cysts are less variable in size and slightly larger on the average than the trophozoites, though some trophozoites are larger than any cysts. Seventy-five cysts measured, range from 7.7 μ–10.8 μ, with an average diameter of 9.4 μ. Only the cysts within the ciliates were measured.

One of the most interesting facts concerning the cysts of this amœba is that, in our material, no single cyst in any ciliate ever contains more than one nucleus. In one opalinid, there are over two hundred cysts, all uninucleate (Fig. 24) and the only cases of more than one nucleus per cyst occur in those outside the ciliates (Figs. 17 and 18). Although division stages have been observed in the trophozoites, our material shows no cyst the nucleus of which is in any phase of mitosis.

TABLE I

Showing percentage infection of opalinids harboring the endamœbæ

Anuran host	Locality	Opalinid infected	Percentage infected in some individual anurans
Bufo cognatus cognatus	Flagstaff, Arizona	*Zelleriella hirsuta*	84
Bufo woodhousii	Near Tuba City, Arizona	*Zelleriella sp.*	19
Bufo woodhousii	Somerton, Arizona	*Zelleriella sp.*	22
Pleurodema bibroni	Near Coquimbo, Chile	*Zelleriella sp.*	91
Bufo marinus	Panama City, Panama	*Zelleriella opisthocarya*	51

The chromatoids of this amœba vary in appearance. Many are splinter-shaped, typical of *E. coli* (Figs. 15, 17, and 24), while others are bar-shaped, typical of *E. histolytica* (Fig. 18). Glycogen vacuoles are present in many of the cysts (Fig. 15).

Percentage Infection within Some Individual Anurans

Table I shows the percentage infection of opalinids harboring the endamœbæ. It is noted that in the 51 per cent infection of *Zelleriella opisthocarya* from *Bufo marinus* from Panama, only 4 per cent of the small and possibly precystic ciliates (averaging 60 μ in length) are parasitized, whereas among the large forms (averaging 230 μ in length), 98 per cent are infected. A possible explanation of this interesting phenomenon is available. It is a well-known fact that, previous to encystment, there is a period in which the opalinids divide very rapidly, giving rise to very small forms which later encyst. It is possible that, before encystment, the opalinids divide so fast that their rate of division is much more rapid than that of the amœbæ inside them. The large and parasitized zelleriellas which originally contain only a few amœbæ would produce, by successive divisions, a number of precystic forms, some with endamœbæ, others without.

TABLE II

Showing anuran hosts, localities, etc. for opalinids known to harbor endamœbæ

Anuran host	Locality	Describer	Opalinid infected
Polypedates maculatus	Ceylon	Metcalf, 1923 (from Dobell's slide)	*Opalina virgula**
Bufo woodhousii	Utah	Metcalf, 1923	*Zelleriella sp.**
Bufo cognatus	Phoenix, Arizona	Metcalf, 1923	*Zelleriella hirsuta**
Paludicola signifera	City of São Paulo, Brazil	Carini, 1933	*Zelleriella brasiliensis*†
Engystoma ovale	City of São Paulo, Brazil	Carini, 1933	*Zelleriella falcata*‡
Bufo marinus	Panama City, Panama	Stabler, 1933	*Zelleriella opisthocarya*
Bufo woodhousii	Somerton, Arizona	Stabler, 1933	*Zelleriella sp.*
Leptodactylus ocellatus	Uruguay	Carini and Reichenow, 1935	*Zelleriella brasiliensis*
Bufo cognatus cognatus	Flagstaff, Arizona	Stabler and Chen, 1936	*Zelleriella hirsuta*
Bufo woodhousii	Near Tuba City, Arizona	Stabler and Chen, 1936	*Zelleriella sp.*
Pleurodema bibroni	Near Coquimbo, Chile	Stabler and Chen, 1936	*Zelleriella sp.*
Hyla nana	Angra dos Reis, State of Rio de Janeiro, Brazil	Stabler and Chen, 1936	*Zelleriella sp.*§
Bufo woodhousii	Utah	Stabler and Chen, 1936	*Opalina woodhousii*§

* Amœbæ described by Metcalf (1923) as "secondary" and degenerating nuclei.

† Changed by Carini and Reichenow (1935) to *Zelleriella paludicolæ*. Amœbæ described by Carini (1933) as *Brumptina brasiliensis*.

‡ Amœbæ described by Carini (1933) as *Brumptina paulista*.

§ From Metcalf's slides.

Anuran Hosts Harboring Parasitized Opalinids and the Geographical Distribution of these Hosts

Metcalf (1923) figured *Opalina virgula* from *Polypedates maculatus* from Ceylon (Fig. 171*c*), a species of *Zelleriella* from *Bufo woodhousii* from Utah (Fig. 98*b*, *e* and *g*) and *Z. hirsuta* from *Bufo cognatus* from Arizona (Fig. 99*c*, *d* and *e;* Fig. 100*c*), containing what he called "secondary" and degenerating nuclei. The objects within those opalinids we now know, as the result of our study of Metcalf's original slides, to be identical with the endamœbæ described here. Carini (1933) described what he believed to be two species of organisms in zelleriellas from *Paludicola signifera* and *Engystoma ovale* from São Paulo, Brazil. These he was unable to identify and created for them the new genus *Brumptina*. These likewise prove to be identical with this *Endamœba* (Stabler, 1933; Carini and Reichenow, 1935). In a recent report on the subject, Carini and Reichenow (1935) have found the amœbæ in *Zelleriella brasiliensis* from *Leptodactylus ocellatus* from Uruguay. These, with the present authors' data, as shown in Table II, constitute all the observations that to our knowledge have been made on these amœbæ.

We have examined frogs and toads from such widely separated localities as Central and South America, Bermuda, the West Indies and a number of places in the United States. From some localities, large numbers of specimens of a given species of anuran have been examined. The same species of anuran host from different regions harbors opalinids infected with *Endamœba* in one locality and not in another. Then, too, from any particular region some individuals of a particular species of anuran contain parasitized opalinids, others do not.

Parasites of the Endamœbæ

In *Pleurodema bibroni* from Chile and *Bufo marinus* from Panama, some of the endamœbæ in the zelleriellas are parasitized by what appears to be a *Sphærita* (Fig. 13). These organisms are small and spherical, from one to fifty occurring in a single amœba. Occasionally they are found in the cytoplasm of the zelleriellas.

Discussion

It seems surprising that, with the exception of the short account by Stabler (1933) and the recent publication by Carini and Reichenow (1935), no other description of this organism has been published in which its true nature has been appreciated.

Carini and Reichenow are correct in believing Metcalf's *Zelleriella hirsuta* from *Bufo cognatus* to contain endamœbæ, but are mistaken in suggesting that the objects in *Zelleriella sp.* from *B. woodhousii* are *Sphærita.* They overlooked Metcalf's figure of the same bodies in *Opalina virgula* (Metcalf, 1923, Fig. 171*c*).

From our examination of Carini's preparations, we can agree with Carini and Reichenow's (1935) assertion that the *Zelleriella* from *Paludicola signifera* contains an *Endamœba*, an opinion recorded by Stabler in 1933. Carini (1933) probably failed to recognize these objects as amœbæ because of poor fixation and staining, but we have found undoubted trophozoites (Fig. 30) and cysts (Fig. 31) within the zelleriellas from his slides.

Unlike our own, Carini's preparations of opalinids from *P. signifera* show an abundance of uni-, bi- and quadri-nucleate cysts free from the ciliates (Figs. 32, 33, 34 and 35). As mentioned by Carini and Reichenow, these cysts present essentially the same morphology as the ones within the ciliates. We have observed, however, an additional feature of these cysts in Carini's preparations not noted by the above workers. The majority of the cysts outside the opalinids possess an enveloping membrane outside of and apart from the cyst wall (Figs. 32, 33, 34, and 35). This was also noted as a feature of *E. ranarum* by Sanders in 1931.

Concerning the trophozoites, Carini and Reichenow (1935) state that, with one exception, they were never found free from the ciliates. In Carini's own material from *P. signifera* the present writers have

EXPLANATION OF PLATE IV

FIG. 25. *Endamœba* trophozoite in *Zelleriella hirsuta* from *Bufo cognatus*, from Metcalf's slide.

FIG. 26. *Endamœba* trophozoite in *Zelleriella sp.* from *Bufo woodhousii*, from Metcalf's slide. Due probably to unsatisfactory technique, the *Endamœba* characteristics are not well shown in this animal.

FIG. 27. *Endamœba* trophozoite in *Opalina woodhousii* from *Bufo woodhousii*, from Metcalf's slide.

FIG. 28. *Endamœba* trophozoite in *Zelleriella sp.* from *Hyla nana*, from Metcalf's slide.

FIG. 29. *Endamœba* trophozoite in *Opalina virgula* from *Polypedates maculatus*, from Dobell's slide.

FIG. 30. *Endamœba* trophozoite in *Zelleriella paludicolæ* from *Paludicola signifera*, from Carini's slide.

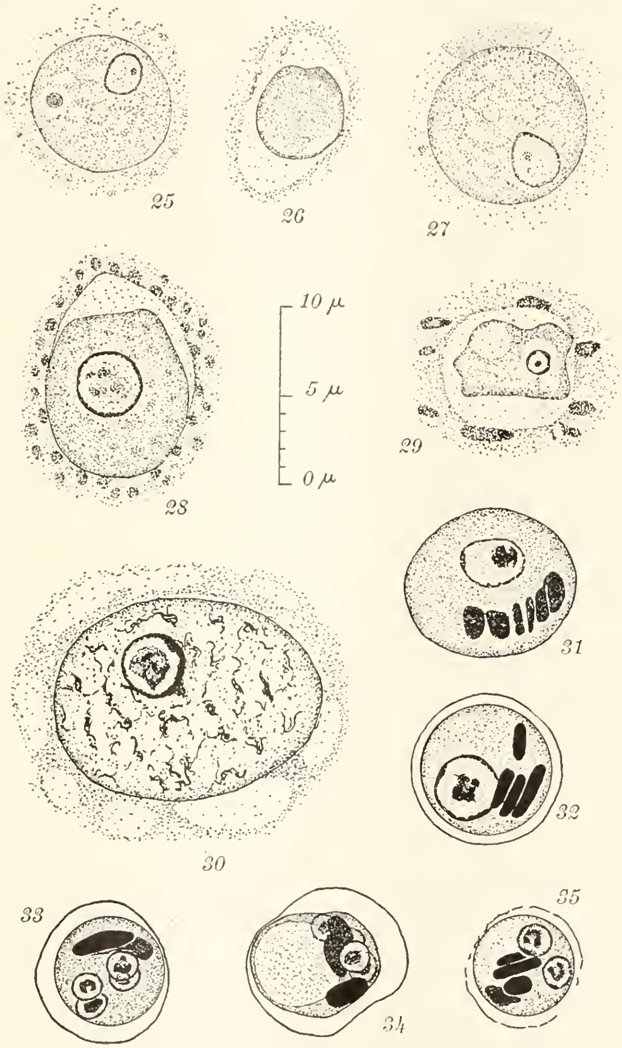
FIG. 31. *Endamœba* cyst in *Zelleriella paludicolæ* from *Paludicola signifera*, from Carini's slide. Compare with Fig. 32.

FIG. 32. *Endamœba* cyst outside the ciliates from *Paludicola signifera*, from Carini's slide. Note the single nucleus and extracystic membrane. Compare with Fig. 31.

FIG. 33. *Endamœba* cyst outside the ciliates from *Paludicola signifera*, from Carini's slide. Note the four nuclei and extracystic membrane.

FIG. 34. Same as Fig. 33.

FIG. 35. *Endamœba* cyst outside the ciliates from *Paludicola signifera*, from Carini's slide. Note the two nuclei and extracystic membrane.


25
26
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10 μ
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Plate IV

observed dozens of amœbæ outside the opalinids. We also cannot support Carini and Reichenow's assertion that no karyosomes can be seen in the nuclei of these amœbæ since this nuclear structure has been found in our material. Then, too, in our slides the amœbæ inside the ciliates are occasionally found filled with the endospherules of the latter, in contrast to the above writers' findings that they were always free of solid food.

Carini and Reichenow believed that, in their material, trophozoites of the amœba invade ciliates already containing the uninucleate cysts and then proceed to phagocytize these cysts. Their figure of this is unconvincing and we have seen nothing in any of Carini's nor in our own slides suggesting this phenomenon.

This brings us to the question of the specific name for this amœba. Carini and Reichenow believed it to be either identical with *E. ranarum* or a race or species derived from it. Stabler (1933) identified it as an *Endamœba*, a decision confirmed by Carini and Reichenow (1935). He gave it the specific name *E. brasiliensis*, since Carini (1933), who gave the first description of these organisms, has designated *B. brasiliensis* as the name of his first species of *Brumptina* (= *Endamœba*). It has since been recalled that Aragão in 1914 used the name *E. brasiliensis* for what he considered to be a new amœba from man. It is generally agreed that Aragão's description deals mainly with *Endamœba coli*, the name therefore being dismissed as a synonym of the latter form. *E. brasiliensis* cannot, then, be revived for the form described here and as Carini designated a second species of *Brumptina*, *B. paulista*, this species name becomes the one available. If this is a distinct species of amœba, its name should then be *Endamœba paulista* (Carini, 1933).

However, at the present time we do not attempt to establish its specific identity, because the question as to its synonymy with *E. ranarum* is still unanswered. It must be admitted that morphologically they are quite similar, presenting the extracystic membrane, rather similar chromatoids, etc. *E. ranarum*, however, is somewhat larger, most writers (Sanders, 1931) stating that it ranges from 10 μ–30 μ in diameter, while the range for the amœba from the opalinids is 5.3 μ–14.3 μ. Also the food of the latter organism seems to be different. *E. ranarum* feeds readily on bacteria and debris in the rectum of the anuran, while these amœbæ, outside the ciliates, are never observed to contain food and those in the ciliates contain ingested endospherules only. In all probability infection experiments will be necessary to finally establish its identity.

Still another question concerns the mode of entrance of the amœbæ into the astomatous opalinids. This process has never been observed by us. It seems probable that one or a few amœbæ may enter a ciliate and the progeny eventually fill the host. They may enter the ciliates when the rectal contents of the frogs and toads are rather dry, this condition preventing freedom of motion on the part of the ciliates. As it has been shown that the opalinids can divide successfully when containing amœbæ and that division stages have been observed in the amœbæ within the ciliates, these, too, may be important factors in carrying on the infection.

It seems possible that the cysts of the endamœbæ which have been freed from the ciliates constitute an infective stage so far as the new anuran host is concerned. They may be deposited in the ponds at the breeding season and be available for ingestion, along with the cysts of the opalinids, by the tadpoles. In the new anuran host we again face the problem of the penetration of the rapidly swimming ciliates by the slowly moving amœbæ. It is also possible that the amœbæ may even be within the encysted opalinids and pass from adult anurans to tadpoles with the latter.

Many other problems concerning this amœba remain yet to be solved. Why do some ciliates contain such large numbers of very small amœbæ and is there any significance to be attached to these small forms? Do the cysts always remain in the uninucleate condition until released from the ciliates? What are the appearances of the prophase and metaphase stages of mitosis? Would some races of undoubted *E. ranarum* invade opalinids?

Summary

1. Observations are presented on an *Endamœba* living in the opalinid ciliates from anuran hosts.

2. Large numbers of frogs and toads from widely separated regions have been examined. The endamœbæ have been found in some of the opalinids collected from the United States (Arizona and Utah), Panama (Panama City), Brazil (states of São Paulo and Rio de Janeiro), Chile (near Coquimbo), Uruguay and Ceylon. In a given locality, only certain species of anurans and a certain percentage of individuals of a given species contain the parasitized opalinids.

3. Within some individual anuran hosts, 91 per cent of the ciliates are infected. There may be a single, a few, many, or over a hundred of these amœbæ within a single opalinid. Usually in any one ciliate all the amœbæ are in the same stage of development, either cysts or trophozoites.

4. The amœbæ seem to produce no serious effect on the opalinids,

as the latter swim actively about in the saline solution and were seen to undergo binary fission even though heavily parasitized.

5. The trophozoites range in diameter from 5.3 μ–14.3 μ, averaging 8.0 μ. They appear to feed on the endospherules of the opalinids and a number of dividing stages have also been observed in the trophozoites.

6. The cysts are less variable in size and are slightly larger on the average (9.4 μ) than the trophozoites, though some trophozoites are larger than any cysts. Only uninucleate cysts have been found within the opalinids although uninucleate, binucleate and quadrinucleate ones were found outside the ciliates. The chromatoids vary in appearance; many are splinter-shaped, while others are bar-shaped. Glycogen vacuoles are present in many cysts.

7. In the zelleriellas from *Pleurodema bibroni* from Chile and in *Bufo marinus* from Panama, some of the endamœbæ were invaded by a *Sphærita*-like organism, which was also observed in the cytoplasm of some of the ciliates.

Acknowledgments

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For live specimens of Anura from Panama, Arizona, Chile, Bermuda and the West Indies, we are indebted to the following: Dr. H. C. Clark of the Gorgas Memorial Laboratory of Panama; Mr. L. M. Klauber of the Natural History Museum at San Diego, California; Dr. H. S. Colton of the Museum of Northern Arizona at Flagstaff, Arizona; Mr. M. G. Netting of the Carnegie Museum at Pittsburgh, Pennsylvania; Messrs. Rafael Varleta and Luis F. Varleta of La Serena, Chile; Dr. J. F. G. Wheeler of the Bermuda Biological Station; and to Messrs. K. V. DuQuesney and Charles DuQuesney of Highgate, Jamaica. For the loan of a number of slides of opalinids we wish to thank Dr. M. M. Metcalf, Dr. Robert Hegner of the Johns Hopkins University and Dr. R. W. Miner of the American Museum of Natural History. Also through the courtesy of Dr. Metcalf, we were able to examine some of the original preparations of Dr. A. Carini and Mr. C. C. Dobell, F.R.S.

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FURTHER STUDIES ON THE ENDAMŒBÆ PARASITIZING OPALINID CILIATES

TZE-TUAN CHEN AND ROBERT M. STABLER

(*From the Osborn Zoölogical Laboratory, Yale University, and the Zoölogical Laboratory, University of Pennsylvania*)

INTRODUCTION

In two previous papers (Stabler, 1933; Stabler and Chen, 1936) we have described in detail the morphology of both the cysts and the trophozoites of amœbæ parasitic in opalinids. In the present note, we wish to report additional genera and species of opalinids infected, the geographical distribution of the amœbæ, and a method of transmission of amœbæ from adult anurans to tadpoles. A summary of this paper has been published as an abstract (Chen and Stabler, 1935).

MATERIAL AND METHODS

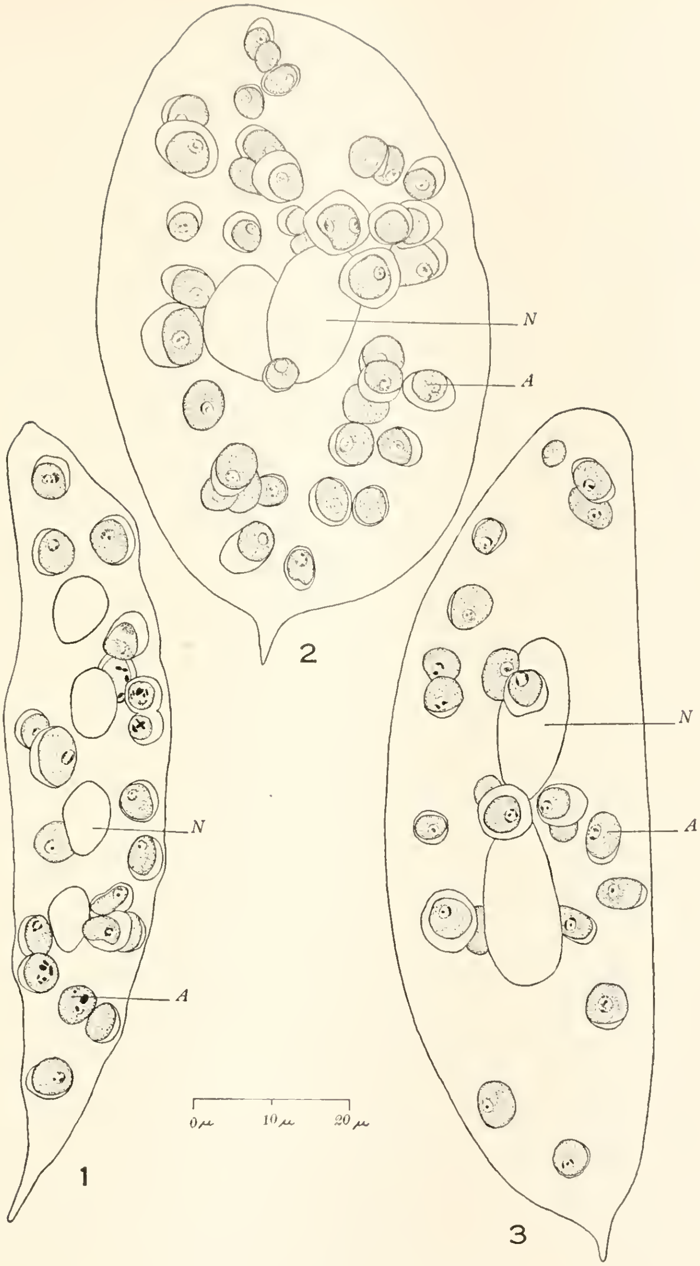
The present report includes observations on the following genera and species of opalinids parasitized by the amœbæ: *Protoopalina stevensoni*, *P. nutti* and *P. regularis*, all in *Bufo regularis regularis* from Cairo; *P. quadrinucleata* in *Rana plancyi* from Peiping; zelleriellas in *Bufo marinus* from several localities in the state of São Paulo; *Cepedea longa* in *Rana limnocharis* from Nanking; and *Opalina woodhousii* in *Bufo woodhousii* from Arizona. A number of other species of opalinids were also examined but found to be free from the parasites.

Most of the material was secured in the following way. The opalinids, after being smeared on the cover glasses, were immediately fixed in warm Schaudinn's fluid containing 5 per cent of glacial acetic acid. This was done in the localities where the different anuran hosts were found and the smears, after being gradually dehydrated, were preserved in 70 per cent alcohol and shipped to us. The ciliates were then stained in Heidenhain's hæmatoxylin, the destaining being carried out mainly in picric acid, although some smears were destained in iron alum.

All drawings were made with the aid of the camera lucida.

OBSERVATIONS AND DISCUSSION

Studies on additional material obtained from distant localities have revealed the fact that the endamœbæ living in the opalinids have a very wide geographical distribution. The animals have been found in some of the opalinids collected in Egypt (Cairo), China (Nanking



EXPLANATION OF PLATES

Drawings all made with the aid of the camera lucida from material stained in Heidenhain's hæmatoxylin. *N* = nucleus of the opalinid host, *A* = the parasitic amœba.

PLATE I

FIG. 1. Trophozoite of *Protoopalina quadrinucleata* containing many amœbæ.
FIG. 2. Trophozoite of *Protoopalina stevensoni* containing many amœbæ.
FIG. 3. Trophozoite of *Protoopalina nutti* containing many amœbæ.

PLATE II

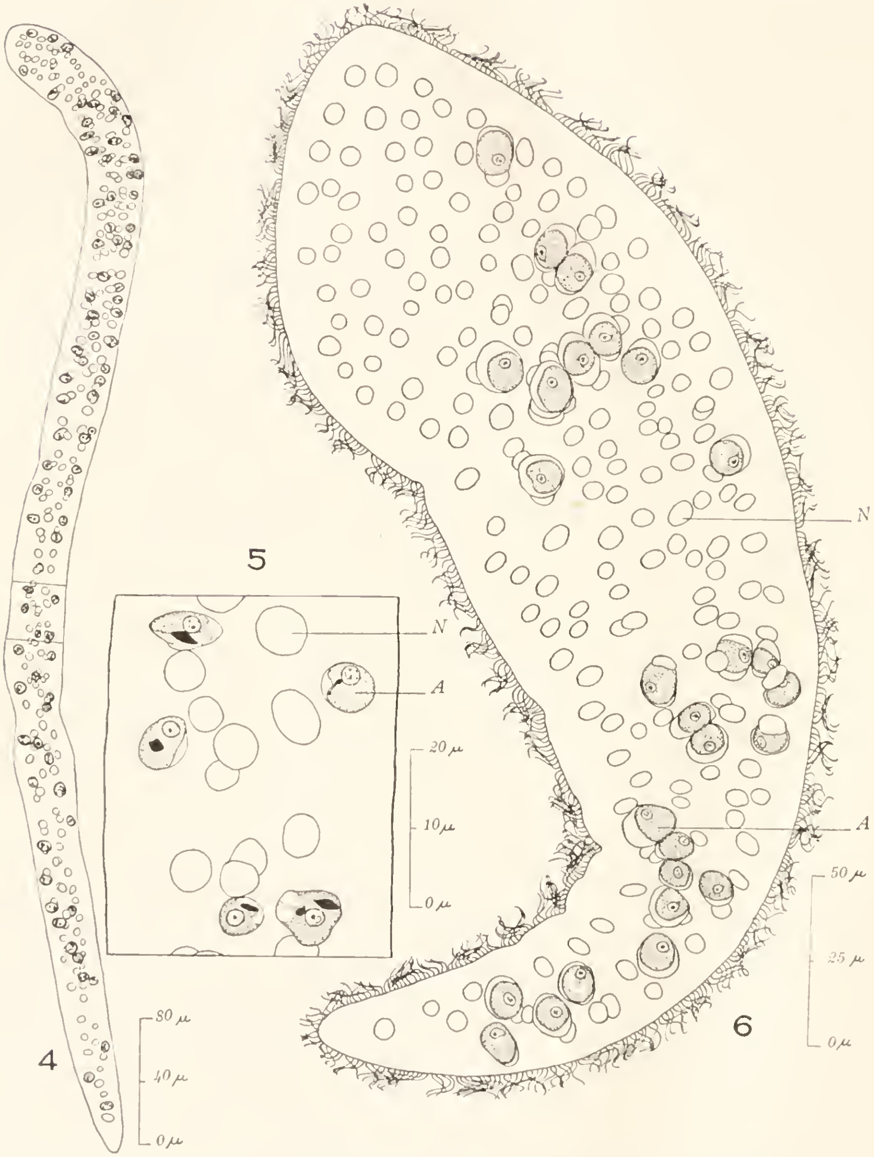

4
80 μ
40 μ
0 μ
5
N
A
20 μ
10 μ
0 μ
6
N
A
50 μ
25 μ
0 μ

FIG. 4. Trophozoite of *Cepedea longa* showing presence of many amœbæ.

FIG. 5. Area from Fig. 4 drawn at a high magnification in order to show the details of the amœbæ.

FIG. 6. Trophozoite of *Opalina woodhousii* showing presence of numerous amœbæ. Cilia diagrammatic.

and Peiping), Ceylon, the United States (Utah and Arizona), Panama (Panama City), Brazil (the states of São Paulo and Rio de Janeiro), Uruguay and Chile (near Coquimbo). The localities cited in our previous paper (Stabler and Chen, 1936) are included in the present list.

The presence of endamœbæ in two genera of the Opalinidæ—*Zelleriella* and *Opalina*—has already been reported by us, but further studies have shown that different species belonging to all four genera of the family may be parasitized (Figs. 1–6). In the previous paper (Stabler and Chen, 1936) there were listed eight species of *Zelleriella* and two species of *Opalina* as being infected and in the present study, four species of *Protoopalina*, one additional species of *Zelleriella*, and a species of *Cepedea* have been found to contain the parasites (Table I).

TABLE I

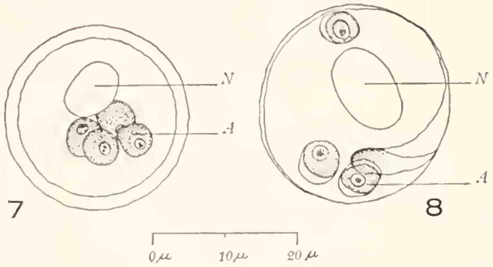
Showing percentage of infection for opalinids harboring endamœbæ. (For a list of other opalinids infected and their percentage of infection, see Tables I and II, Stabler and Chen, 1936.)

Anuran host	Locality	Opalinid infected	Percentage infected in some individual anurans
Bufo regularis regularis	Cairo, Egypt	*Protoopalina stevensoni*	7
Bufo regularis regularis	Cairo, Egypt	*Protoopalina nutti*	39
Bufo regularis regularis. . . .	Cairo, Egypt	*Protoopalina regularis*	34
Rana plancyi	Peiping, China	*Protoopalina quadrinucleata*	2
Bufo marinus	Taubaté, State São Paulo, Brazil	*Zelleriella sp.*	100
Rana limnocharis	Nanking, China	*Cepedea longa*	*
Bufo woodhousii	Near Tuba City, Arizona	*Opalina woodhousii*	50

* Percentage infection in this form could not be determined accurately because of the small number of opalinids available.

As noted before, in the different localities, only certain species of anurans and a certain percentage of individuals of a given species harbor the parasitized opalinids. For example, out of 37 frogs and toads (*Rana mascareniensis mascareniensis* and *Bufo regularis regularis*) examined at Cairo, 35 have been found to contain the opalinids. Among these 35 specimens, only five (all *Bufo regularis regularis*) contain opalinids parasitized by amœbæ, whereas others are free from the parasites. There is a great range of variation in the percentage infection of opalinids among individual anuran hosts (Table I). The zelleriellas in some individuals of *Bufo marinus* from Taubaté, State of São Paulo, Brazil, are 100 per cent infected, whereas in *Rana plancyi* from Peiping only a few specimens of *Protoopalina quadrinucleata* contain any amœbæ. It is difficult to explain such an unusually low percentage of infection in this *Protoopalina*.

In some anuran hosts, when their opalinids are very heavily parasitized, the small, precystic forms also contain numbers of amœbæ. The encysted ciliates, when present in these anuran hosts, may also harbor the parasites. In the preparations of *Protoopalina nutti* in *Bufo regularis regularis* from Cairo and zelleriellas in *Bufo marinus* from Taubaté, amœbæ have been observed within the opalinid cysts (Figs. 7 and 8), lying in the cytoplasm of the ciliates. In the opalinid cysts only trophozoites of the amœba have been found. Such parasitized encysted opalinids were particularly numerous in the *Bufo marinus* material; in one individual host nearly 100 per cent of the cysts were infected. These cysts contain from one to seven amœbæ, although the majority harbor only two, three, or four of them. On one slide, for example, out of 205 cysts taken at random, 67 contain 3 amœbæ per cyst, 65 contain 2, 32 contain 4, 23 contain only one, 15 contain 5, 2 contain 6, and only one cyst contains none.



Figs. 7 and 8. Cysts of opalinids containing amœbæ.
Fig. 7. Cyst of *Zelleriella sp.* from *Bufo marinus* containing four amœbæ.
Fig. 8. Cyst of *Protoopalina nutti* containing three amœbæ.

It seems likely that the presence of amœbæ within the encysted opalinids constitutes an important method of transmission of amœbæ from adult anurans to tadpoles, thus establishing infections in the new generation of anurans. During the breeding season of frogs and toads, the opalinid cysts containing the amœbæ may be extruded and deposited in the ponds. The young tadpoles, browsing at the bottom of the pond, may ingest the parasitized opalinid cysts together with diatoms, other algæ, and debris. The encysted opalinids (carrying the amœbæ in their cytoplasm), after reaching the intestines of the tadpoles, escape from the cysts and later develop into trophozoites, probably still containing the amœbæ. Thus the amœbic infection of the opalinids may become established in these young anurans.

SUMMARY

1. The amœbæ parasitic in the opalinid ciliates have a very wide geographical distribution, being found in Egypt, China, Ceylon, the United States, Panama, Brazil, Uruguay and Chile.

2. Different species belonging to all the four genera of the family Opalinidæ—*Protoopalina*, *Zelleriella*, *Cepedea*, and *Opalina*—have been found parasitized by the amœbæ.

3. There is a great range of variation in the percentage infection of opalinids in different individual anuran hosts. In some hosts it may be as low as 2 per cent, whereas in some others 100 per cent of the opalinids may be infected.

4. The amœbæ have been found within numerous opalinid cysts. Each cyst may contain one to seven amœbæ although the majority of cysts harbor only two, three, or four of them. The presence of amœbæ in the encysted opalinids probably constitutes an important method of transmission of amœbæ from adult anurans to tadpoles.

Acknowledgments

This study was aided by a grant from the Bache Fund, National Academy of Sciences, and was carried on while one of us (T. T. C.) was holding a Sterling Research Fellowship at Yale University. We are also indebted to Dr. D. H. Wenrich for his helpful advice. We wish to express our thanks to Professor H. Sandon, formerly of the Egyptian University at Cairo, Professor J. C. Li, and Mr. C. S. Lin of Yenching University at Peiping, and Professor S. B. Pessoa of Faculdade de Medecina de São Paulo, Brazil, for large numbers of well-fixed smears of opalinids from these different localities; and to Mr. Da-Shu Nie of the Science Society of China at Nanking for the loan of a number of slides.

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THE EARLY DEVELOPMENT OF ASCIDIAN EGGS

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Introduction

The eggs of ascidians have been a favorite material for investigation for many years. Owing to the presence of extra-oval membranes, observations on the nude egg were not possible. This difficulty was finally overcome by the method of Berrill (1932), who observed that hatching was partially effected by the digestion of the membranes and experimented to discover a readily accessible source of suitable enzymes. Effective enzymes he found were contained in the stomach juices of certain Crustacea, for example the crab, *Munida*. As a result of this discovery, it is now possible to obtain any desired quantity of nude eggs.

Materials and Methods

The observations described below were made on the egg of the ascidian, *Ascidiella aspersa*, obtained in the vicinity of Plymouth, either at Mill Bay Docks or at the Salstone (Salcombe) and *Phallusia mammillata* obtained at the Salstone during the months of May and June, 1935. As soon as was possible the ascidians were placed in basins through which sea water was circulated. Under these conditions eggs capable of producing larvæ could be obtained for at least several days. Of the stains used—Clark-Lubs sulphthalein group, Nile Blue Hydrochloride and Neutral Red—only the latter two were found able to penetrate the egg. The Clark-Lubs group will not penetrate even after several hours, nor will they penetrate when the medium is changed to isotonic NaCl solution.

To obtain the eggs the integuments overlying the oviduct were removed carefully with a small pair of forceps, the oviduct punctured with a sterile steel needle, the eggs withdrawn with a pipette and placed at once in a small glass dish containing sterile filtered sea water. The egg membranes were then removed with the stomach juice of the crab

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[1] The observations recorded here were made at the Laboratory of the Marine Biological Association of the United Kingdom, while holding the Moyse Travelling Scholarship of McGill University. I wish to express my gratitude to Professor J. Stanley Gardiner, F. R. S., for his kind permission to occupy the Cambridge Table, and to Dr. E. J. Allen, F. R. S., and the staff of the Laboratory for their willing and courteous coöperation at all times.

Munida, according to the method described by Berrill (1932). With a dilution of one part *Munida* stomach juice to fifty parts sea water the membranes were readily digested off and the previously floating eggs then sink to the bottom of the dish. The resulting nude eggs when washed with fresh sea water were capable of giving perfect development.

The stains were made up in 0.5 per cent concentration in distilled water. About one drop was sufficient to give a faint coloration to 15–20 cc. of sea water, into which the nude eggs were then introduced. Ten to fifteen minutes were sufficient to give the egg a distinct and characteristic stained appearance. The eggs were then transferred to fresh sea water; they retained the stain throughout the development of the egg.

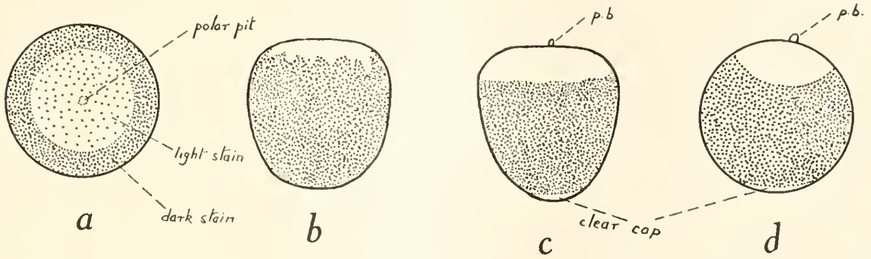

FIG. 1. Nude egg of *Ascidiella aspersa* vitally stained with Nile Blue. The dots indicate the limits and density of the stain. (*a*) unfertilized egg; (*b*) 18 minutes after insemination; (*c*) 21 minutes after insemination—the first polar body has been extruded; (*d*) 24 minutes after insemination, the egg has returned to a spherical condition.

Sperm was obtained directly from the spermduct in a manner similar to that described above for obtaining the eggs. Fertilization was effected by adding the sperm to the dish containing the stained eggs. After about five minutes the eggs were washed free from sperm.

A binocular dissecting microscope was used and when necessary a camera lucida. All observations recorded were made on the living egg.

Behaviour of Stain from Fertilization to First Polar Body

The appearance of a stained unfertilized egg of *Ascidiella aspersa* is shown in Fig. 1, *a*. There is present a peripheral deeply-stained layer extending inward for about one-third of an egg radius. The central part of the egg stains much less intensely and is well marked off from the peripheral zone. This is even more strikingly evident in

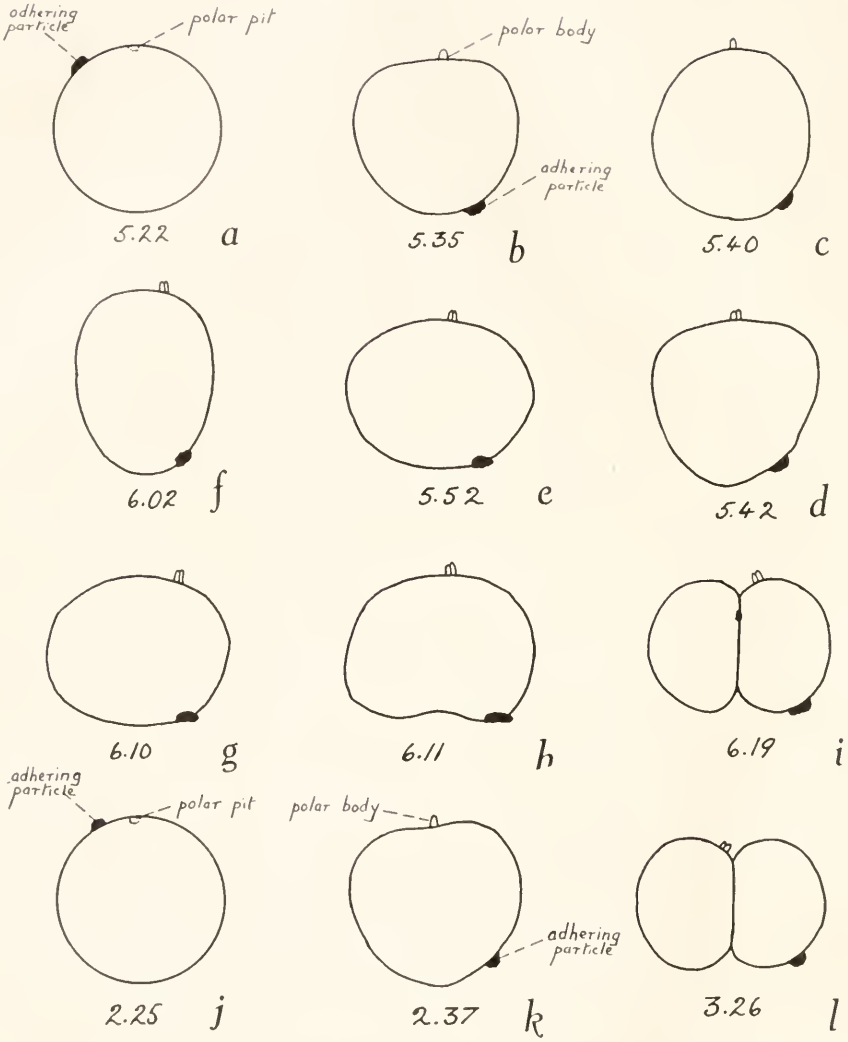
adhering particle
polar pit
5.22 a
polar body
adhering particle
5.35 b
5.40 c
6.02 f
5.52 e
5.42 d
6.10 g
6.11 h
6.19 i
adhering particle
polar pit
polar body
adhering particle
2.25 j
2.37 k
3.26 l

FIG. 2. Nude eggs of *Ascidiella aspersa* following fertilization showing the relationship between an adhering particle and the polar-pit, and the same particle to the first polar body. The figures indicate the time in hours and minutes of the observations. (*a*)–(*i*) records of one egg; (*j*)–(*l*) records of a second egg.

the egg of *Phallusia*. The egg presents the same appearance from all aspects with but one exception. This is the presence of a minute, unstained area, the "polar pit" ("la tache polaire," Dalcq, 1932), on the periphery of the egg, which shows up clearly against the stained background. About 18 minutes after insemination the egg loses its spherical shape and becomes somewhat flattened in the region of the "polar pit." At the same time the stain disappears from the region of the egg just beneath the flattened part (Fig. 1, *b*). The flattening and loss of stain occur very rapidly, requiring only a few seconds. Coincident with these changes the stain in the remainder of the egg no longer remains distinctly separated into peripheral and central regions but assumes a homogeneous distribution. About two minutes after these changes the first polar body is cut off from the clear flattened area. The egg has become somewhat elongated in what can now be identified as the animal-vegetal axis (Fig. 1, *c*). At the same time it can be seen that there is present a thin peripheral layer in the vegetal pole which is clear and unstained. From its position this clear cap seems to correspond to the "milky or yellowish crescent" observed by Tung (1932) on this egg, and possibly to the yellow crescent in the egg of *Styela partita* (Conklin, 1905). A few minutes after the polar body is extruded the egg returns to a spherical condition and the stained and unstained areas are clearly seen (Fig. 1, *d*).

DETERMINATION OF THE SITE OF POLAR BODY FORMATION

Dalcq (1932) has described the "polar pit" as the site of formation of the second polar body, and adds in a footnote (p. 229) that he has never observed more than one polar body ("globule") cut off. The latter statement is contrary to the present authors' observations, for numerous eggs were observed with two or even three polar bodies present, the latter condition no doubt indicating that the first polar body had divided. However, the polar bodies are very readily knocked off when the egg is manipulated and it is only rarely that all three polar bodies are seen on the egg. Furthermore, the method employed by Dalcq to remove the egg membranes, by the use of finely pointed forceps (p. 227, *op. cit.*), is doubtless a tedious and laborious method so that the number of eggs investigated by this author was probably small, whereas by the method of digesting off the membranes as was used in the present investigation, hundreds of nude eggs were easily obtained for observation. Nor was it possible to confirm the statement that the polar-pit is the site of the second polar body (or of the first). As was mentioned above, the area containing the unstained polar pit loses its stain shortly *before* the polar body is cut off so that it was not possible to observe the relationship between polar-pit and polar-body directly. However, if the relationship between the polar-pit and some fixed point on the egg could be determined before the polar-body were formed, and this same fixed point compared with the newly-formed polar-body, the polar-pit and polar-body should both bear the same relationship to the fixed point on the egg, if there is an identity of site between them. This was tested in the following manner.

Unfertilized nude eggs of *Ascidiella aspersa* are stained with Nile Blue. After washing the eggs, sperm was added, about five minutes allowed to elapse, the eggs washed free from sperm and placed in a dish under a binocular microscope. Then it can be seen that particles have adhered to some of the eggs. An egg to which a suitable particle has become attached is selected and kept in the centre of the field, and a camera lucida drawing is made showing the position of the adhering particle and the polar-pit. Upon the appearance of the first polar-body another drawing is made to show the position of the particle and the polar-body, and the drawing compared with the previous one. Figure 2, *a–i* shows a series of drawings of the same egg from five minutes after insemination to the first cleavage. Inspection of these drawings reveals at once that the small angle formed by lines joining the adhering particle and polar-pit to the centre of the egg does not compare with the considerably larger angle formed by the lines joining the particle and polar-body to the centre of the egg. Figure 2, *j–l* shows another egg in which again the above-mentioned condition obtains. Observations on several other eggs gave similar results. In one case the angle before and after polar-body formation was approximately the same, but this is to be expected occasionally if, as the above drawings suggest, there is no relationship between the site of the polar-pit and the polar-body.

Now, as will be described below, the egg undergoes a series of changes in shape from just before the formation of the polar-body to the time of first cleavage (Fig. 3). It might be expected, then, that the apparent change in the angle before and after polar-body formation was due to movement of the particle over the surface of the egg due to movement of the surface layer of the egg to which it adheres. But this is hardly likely to be the case, since the change in shape which the egg undergoes before polar-body formation is a simple flattening and elongation, whereas from the time of polar-body formation to first cleavage the egg undergoes many and varied changes, yet during this period the relationship between particle and polar-body remains the same, as is seen from Fig. 2, *c–i*.

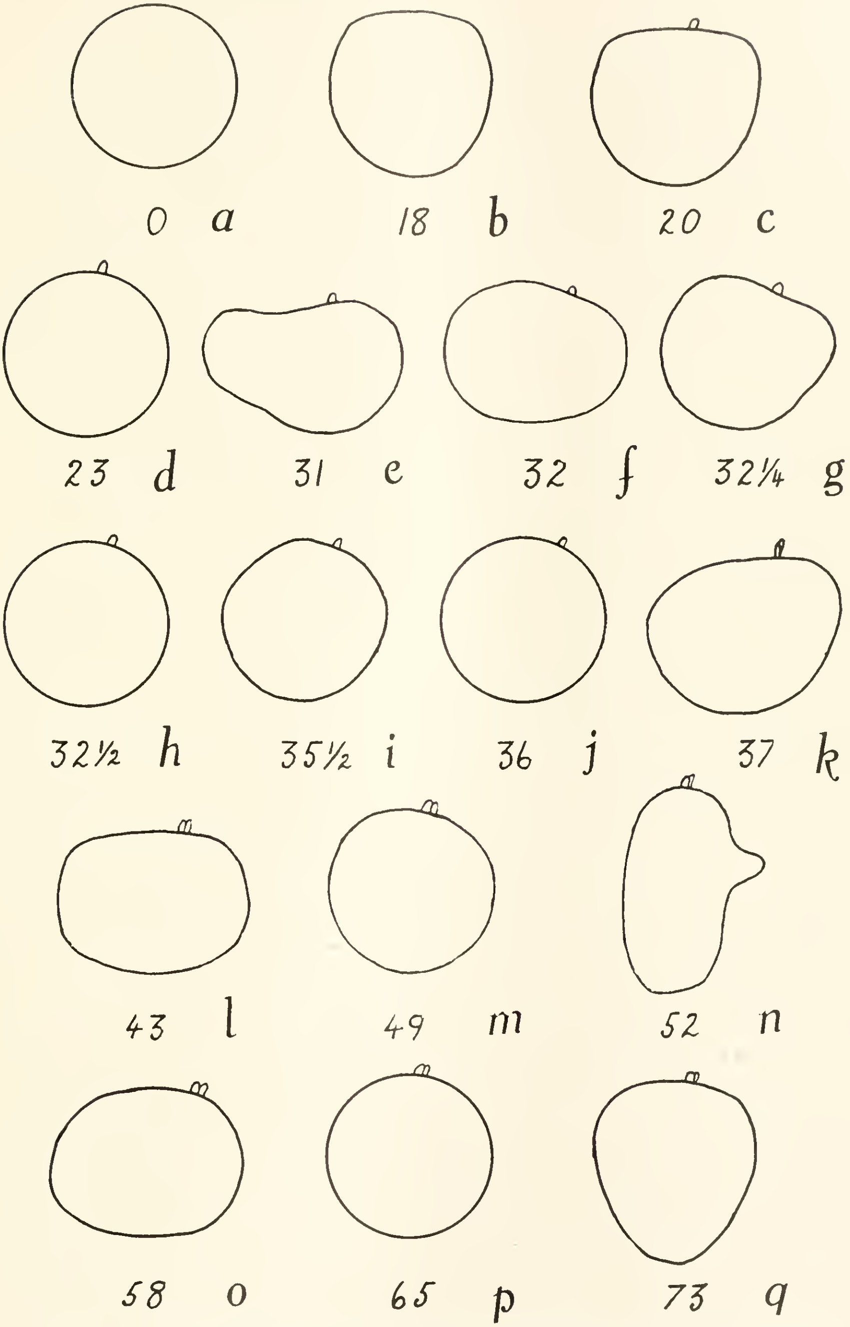
0 a
18 b
20 c
23 d
31 e
32 f
32¼ g
32½ h
35½ i
36 j
37 k
43 l
49 m
52 n
58 o
65 p
73 q

FIG. 3. Changes in shape of the nude egg of *Ascidiella aspersa* from fertilization to just before first cleavage. The figures indicate the time in minutes following insemination.

Since Dalcq accepted the site of the polar-pit as being identical with that of the polar-body and used it to orient the eggs for cutting, it follows that some doubt is thrown on the validity of his conclusions regarding the localization of presumptive germinal regions in the unfertilized egg of *Ascidiella aspersa*, although, of course, not on the existence of such presumptive regions.

Changes in Egg Shape from Fertilization to First Cleavage

Changes in the shape of an egg from the time of insemination to the first cleavage have been recorded. Recently Hoadley (1934) has given an account of such changes in the egg of *Nereis* and has related the movements to definite intervals from insemination to first cleavage, finding that the most marked changes in shape occur from the time of insemination to the breakdown of the germinal vesicle, but after being interrupted by "rest intervals" the changes continue to the time of first cleavage. Whereas the above observations were made on the egg of *Nereis* enclosed in its membrane, the observations recorded here were made on nude eggs.

The egg of *Ascidiella aspersa*, when removed from the oviduct, is enclosed by two membranes, separated by a space. The inner membrane adheres closely to the surface of the egg so that when development occurs with these membranes intact no changes in shape of the egg can be observed. However, if a nude egg be observed from the time of insemination to first cleavage, definite and marked changes in shape may be seen. The egg remains spherical until within about two minutes of polar-body formation, when it becomes flattened and somewhat elongated. This elongation is even more marked immediately following the extrusion of the polar-body, which occurs about twenty minutes after insemination (Fig. 2, *a–c*). About three minutes after the first polar-body is cut off, the egg returns to a spherical shape (Fig. 3, *d*) but this condition is not retained for long, the egg then undergoing a series of deformations. Figure 3, *e–i* shows the changes which occur from 31 to 35½ minutes after insemination. One half-minute later the egg has returned to a spherical condition (Fig. 3, *j*). A minute or two later the egg again loses its spherical shape, flattening at the animal pole, and the second polar body is cut off (Fig. 3, *k*). From the time of formation of the second polar-body to first cleavage the egg continues to undergo changes in shape, returning to a spherical or nearly spherical condition from time to time. Figure 3, *l–q* shows the changes in shape in the latter period at intervals of a few minutes.

The cause of these changes was not investigated experimentally. Unlike the egg of *Nereis*, the germinal vesicle of the egg of *Ascidiella aspersa* is ruptured upon entering the oviduct, hence before fertilization. Consequently the changes in egg shape cannot be referred to the rupture of the germinal vesicle. Nevertheless, it appears likely that the changes in shape are associated with a transitory decrease in viscosity of the protoplasm. At the same time the surface membrane of the egg must possess considerable elasticity—despite its great delicateness, as is evidenced by the facility with which it is ruptured upon handling—to be able to undergo such changes as are shown, for example, in Fig. 3, *n*.

History of Stain from First Polar Body to Tadpole

After the first polar body has been extruded and the egg has returned to a spherical condition, there is present a clear unstained cap of protoplasm at the animal pole and a thinner clear protoplasmic cap at the vegetal pole; the remainder of the egg appears to be homogeneously stained (Fig. 4, *a*). Some ten odd minutes before first cleavage the centre of the stained egg becomes less deeply stained and finally almost clear, and is the first indication that the nucleus is preparing to divide. This clear area extends across the egg at right angles to the plane of the first cleavage, and at the same time the beginning of cytoplasmic cleavage becomes evident (Fig. 4, *b–c*). During these changes which lead to the first cleavage it is not possible to identify definitely the clear vegetal cap, but with the formation of the two-cell stage it is seen that it has divided so that each of the two blastomeres possesses a clear cap of protoplasm at the vegetal pole (Fig. 4, *d*). The latter figure also shows that the stain has become largely restricted to the vegetal part of the egg, only extending towards the animal pole, but not completely covering it, as a thin pheripheral layer. After the second cleavage the clear cap of the vegetal pole can no longer be followed. Figure 4, *e* shows a four-cell stage from the animal pole; the stain appears to be distributed about the periphery of each of the blastomeres with the exception of a narrow neck of unstained protoplasm joining the clear central mass of protoplasm of the anterior and posterior blastomeres of each side. The stain is not as intense in the anterior blastomeres and in some specimens appears not to cover completely the animal portion of the anterior blastomeres. Probably the correct interpretation of the discrepancy in the distribution of the stain in the anterior blastomeres is that the stain is present in the vegetal part but not in the animal part.

In the eight-cell stage (Fig. 4, *f*) the four animal blastomeres are

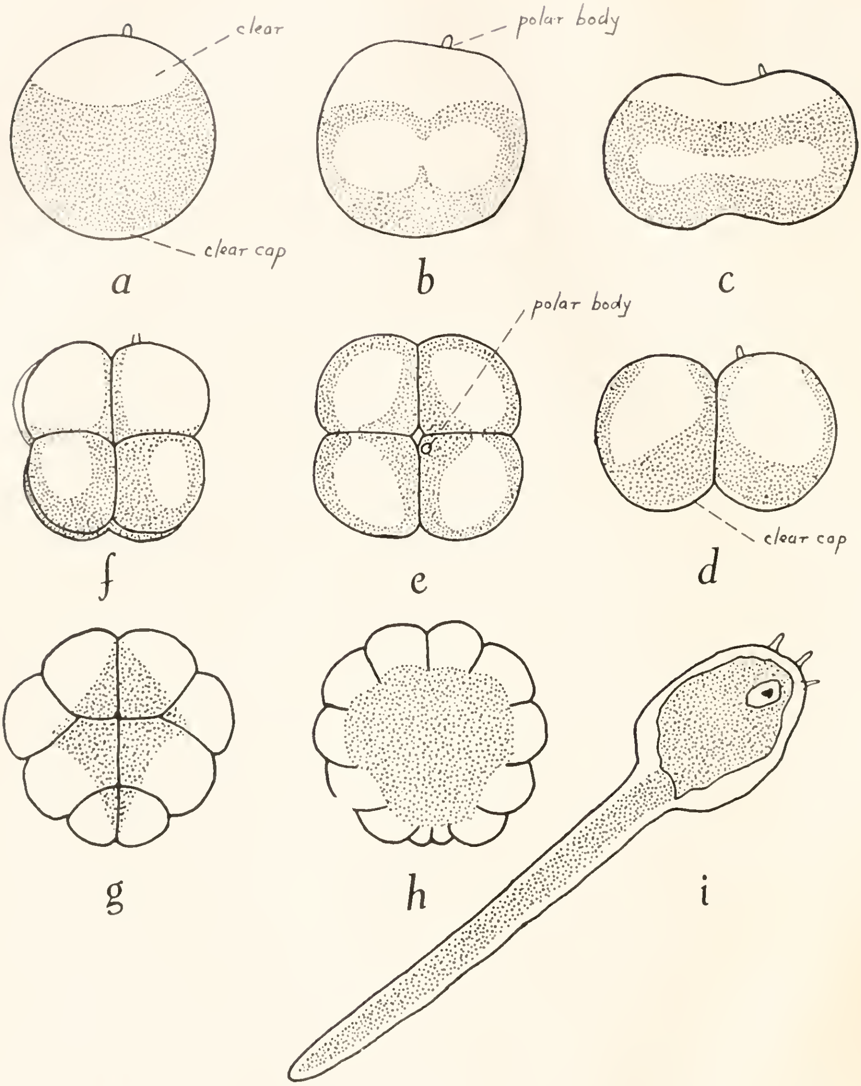
clear
polar body
clear cap
a
b
c
polar body
f
e
d
clear cap
g
h
i

Fig. 4. Nude egg of *Ascidiella aspersa* vitally stained with Nile Blue. The dots indicate the distribution of the stain. (*a*) about three minutes after the first polar body has been extruded; (*b*)–(*c*) early stages of first cleavage; (*d*) two-cell stage; (*e*) four-cell stage from the animal pole; (*f*) eight-cell stage, (*g*) sixteen-cell stage, viewed as a transparent object; (*h*) approximately sixty-four-cell stage, viewed as a transparent object; (*i*) twenty-four-hour tadpole.

almost devoid of stain, which is mainly confined to the vegetal blastomeres. Figure 4, *g–h* shows a 16-cell stage and an approximately 64-cell stage respectively, viewed as transparent objects. It is evident that the stain has become almost entirely restricted to the future endodermal and chordal regions. This conclusion is borne out by the distribution of the stain in the 24-hour tadpole (Fig. 4, *i*), from which it will be seen that the stain is present in the endoderm (gut) and the notochord, the latter being somewhat less densely stained than the former.

Summary and Conclusions

1. The observations recorded are on the living nude eggs of *Ascidiella aspersa* and *Phallusia mammillata*, as obtained in the vicinity of Plymouth and stained with Nile Blue and Neutral Red.

2. In the unfertilized egg the periphery of the egg stains more intensely than the central region, the boundary between the two regions being usually well-defined. One minute area of the periphery remains clear and unstained—this is the so-called "polar pit."

3. The statement by Dalcq (1932) that the "polar pit" marks the site of the second polar body could not be confirmed. On the contrary, evidence is presented which strongly indicates that there is no relationship between the site of the "polar pit" and the site of extrusion of the polar body. This observation throws doubt on Dalcq's conclusions regarding the topography of presumptive germinal regions in the unfertilized egg, though, of course, not on their existence.

4. The changes in shape which the egg undergoes from the time of insemination to first cleavage are recorded. The changes start with a flattening of the egg about two minutes before the first polar body is extruded. About three minutes after the latter event the egg returns to its spherical shape. Shortly afterwards the egg undergoes a series of deformations which last until the first cleavage, being interspersed with periods during which the egg returns to a spherical condition. The changes in shape indicate that the extremely delicate egg membrane nevertheless possesses a high degree of elasticity.

5. Just before the first polar body is cut off the distribution of the stain changes. With the formation of the first polar body it is seen that there is present a cap of clear unstained protoplasm at the animal pole, and a thin cap of clear unstained protoplasm at the vegetal pole. In the young tadpole the stain is found to be confined to the endoderm (gut) and notochord. It seems probable that the movements of the stain indicate the changes which the presumptive chordo-endoplasm undergoes from the unfertilized egg to the time when it becomes definitive endoderm and notochord in the young tadpole.

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STUDIES OF THE MITOTIC FIGURE

V. The Time Schedule of Mitotic Changes in Developing Arbacia Eggs

HENRY J. FRY

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Purpose of the Study

In order that the physiological variations so frequently studied in developing *Arbacia* eggs may be related to the accompanying cytological changes, this paper reports the time schedule of the mitotic events from fertilization to third cleavage.

It is well known that the presence of pigment granules in the living eggs of *Arbacia punctulata* makes it difficult to determine the exact time when many of the cytological changes occur. The time schedule can be worked out completely only in fixed eggs. The facts thus obtained, however, can readily be applied to experiments with living eggs, because, in any given egg-set, as will be demonstrated later, there is a constant relation between the time when it divides and when each mitotic stage occurs. In other words, one can subject *Arbacia* eggs to experimental treatment at various intervals after fertilization, and, having determined the cleavage time, can by the use of such a time schedule calculate the mitotic stage which was present at the time of treatment.

The Mitotic Time Schedule of Arbacia Eggs

The egg-set of one female was employed in the present study, and eggs were fixed at close intervals with Bouin's reagent. The temperature was kept at 20° ± 0.2. The material was sectioned at a thickness of 5 μ, and stained with Heidenhain's hæmatoxylin. At each interval of fixation 100 eggs, chosen at random, were studied at a magnification of 600 ×, to determine the mitotic phases present.

The data are presented numerically in Table I, and in graph form in Chart 1. Photographs of living eggs are also shown, in Plate I.

In making a mitotic time schedule, the manner in which the various stages are defined is an important factor. For example, in the present study telophase (Fig. 8) is considered as beginning when the solid anaphase chromosomes have become numerous small vesicles, and as ending when they have fused to form a single nucleus; the resting phase (Fig. 9) begins there and ends when the prophase chromosomes appear. During both phases the asters gradually fade; they are less clearly organized during telophase than during anaphase; and they become exceedingly vague during the resting condition, finally disappearing just as the new prophase asters arise. However, these two phases might also be defined in terms of changes in the asters, as well as in the nuclear materials, in which case both might be regarded as

TABLE I

The mitotic time schedule of Arbacia eggs from fertilization to third cleavage. The eggs of but one female were used. The temperature was 20° C. ± 0.2. One hundred eggs are reported at each interval of fixation (Bouin's fluid); hence the number of eggs in each phase is its percentage of the total. The figures referred to are shown in Chart 1.

Pronuclei and sperm-aster

Minutes after fertilization	2½	5	8½	11	13	16	20	25			
Nuclei not touching (Fig. 1)	97	92	79	47	8	4					
Nuclei touching (Fig. 2)	3	8	21	53	90	60	19	3			
Nuclei fused (Figs. 3 and 4)					2	36	81	97			

First-cleavage figure

Minutes after fertilization	30	35	40	45	50	55	60	65	70	75	80
Nuclei fused (continued)	98	93	90	60	14	4					
Prophase, early (Fig. 5)	2	7	10	40	40	20					
Prophase, late					46	37	8	6			
Metaphase (Fig. 6)						39	55	7			
Anaphase, early							34	20			
Anaphase, late (Fig. 7)							3	32	6		
Telophase, early								35	20		
Telophase, late (Fig. 8)									66	6	4
Resting (Fig. 9)									8	94	90
Next prophase, early											6

Second-cleavage figure

Minutes after fertilization	85	90	95	100	105	110	115	120	125		
Resting (continued)	62	30									
Prophase, early (Fig. 10)	36	58	35	8							
Prophase, late	2	12	43	30	5						
Metaphase (Fig. 11)			22	45	25						
Anaphase, early				11	22	4					
Anaphase, late (Fig. 12)				6	26	18	4				
Telophase, early					13	18	12	2			
Telophase, late (Fig. 13)					7	42	30	10	6		
Resting (Fig. 14)					2	18	54	84	76		
Next prophase, early								4	18		

TABLE I (cont'd)

Third-cleavage figure

Minutes after fertilization	130	135	140	145	150
Resting (continued)	18	2			
Prophase, early (Fig. 15)	54	46	7		
Prophase, late	22	26	18	4	
Metaphase (Fig. 16)	6	19	51	11	3
Anaphase, early		6	14	12	5
Anaphase, late (Fig. 17)		1	10	38	12
Telophase, early				21	29
Telophase, late (Fig. 18)				11	18
Resting				3	31
Next prophase, early					2

First Cleavage	Minutes after fertilization	67	68	69	70	71	72	
	Percentage of cleaved eggs	20	29	58	69	76	85	
Second Cleavage	Minutes after fertilization	104	105	106	107	108	109	110
	Percentage of cleaved eggs	10	23	34	54	64	66	76
Third Cleavage	Minutes after fertilization	139	141	143	145	146	148	150
	Percentage of cleaved eggs	3	10	34	39	66	81	88

different periods of telophase, the time when the asters are disappearing. In that event the resting phase would be regarded as late telophase, and the shape of the telophase curve would differ from that here reported.

There are difficulties in applying any given set of definitions of mitotic phases to the division figures in various cell types, because asters may be present or absent, the resting period may be long or practically non-existent, and there may be other variations. For this reason the definitions of the mitotic phases used in the present work are given here.

The history of the pronuclei and the sperm-aster falls into three periods: first, when the pronuclei are not yet in contact and the aster is small, delicate, and round; second, when the pronuclei are touching but have not yet fused, and the aster is medium in size but more distinct; and third, when the pronuclei have fused but the prophase configuration has not yet appeared. This is a long period, during which the identity of the male pronucleus is gradually lost in the female pronucleus, and the large round aster becomes sharply elongate and then slowly disintegrates. In *Arbacia* eggs the history of the sperm-aster is atypical in two respects: first, during the late history it is crescent-shaped instead of round; and second, this stage persists an unusually long time. On the other hand, the first-cleavage figure of *Arbacia* eggs is typical of those of most invertebrate eggs.

Prophase, early, begins when the chromosomes first appear as vaguely-defined, thread-like structures which stain darkly; the nuclear membrane is still intact. At this time two new small asters arise. The period ends when the nuclear membrane dissolves.

Prophase, late, begins with the dissolution of the nuclear membrane. The well-formed chromosomes are irregularly scattered on the spindle, but have not yet become aligned in the metaphase plate. During this time the asters increase in size and distinctness.

Metaphase covers only the period when the chromosomes are definitely aligned in the metaphase plate. During this time the asters enlarge slightly and become somewhat more distinct.

Anaphase, early, begins with the separation of the metaphase chromosomes and ends when they have moved about halfway toward the spindle-ends.

Anaphase, late, begins with the latter condition, and ends when the chromosomes have reached the spindle-ends, still in the form of typical densely staining rods. During anaphase the asters attain their maximum size and distinctness.

Telophase, early, begins with the vacuolization of the chromosomes, accompanied by their reduced capacity to stain with Heidenhain's hæmatoxylin. It ends when the fusion of the vacuoles has proceeded about halfway.

Telophase, late, includes the period of the final fusion of the vesicles, and ends when the single rounded nucleus is fully formed. During telophase the aster begins to disintegrate; the fixed rays are more irregular in shape and less distinct than those of anaphase.

The resting stage covers the period when the nuclear materials have the form of a typical nucleus; none of the substances stain with Heidenhain's hæmatoxylin. During this time the asters continue to fade; they disappear about the time when the next prophase asters arise.

The method of defining mitotic phases is not the only cause of discrepancy in making mitotic time schedules. If counts are made only at five-minute intervals, the exact point when a given phase occurs in greatest numbers may not be ascertained; again, it is difficult to decide to which phase to assign an egg in a transitional stage between two phases; furthermore, if not more than 100 cells are counted at each interval, a certain error is involved.

Variation in the Mitotic Time Schedule of Arbacia Eggs

The variability in behavior among the egg-sets of different *Arbacia* females is well known. ". . . freshly shed eggs, from freshly collected individuals, vary widely in a number of morphologic and physiologic traits, such as size, color, shape of eggs, amount of jelly, duration of agglutination, rate and manner of membrane formation, rate and percentage of segmentation, etc." (Goldforb, 1929, p. 350.)

The time of cleavage of the egg-sets of 88 females is presented in Table II. As these data show, there is a variation of about 10 per cent in the cleavage time of different egg-sets obtained from females collected at the same time and place, fertilized simultaneously, and allowed to develop under identical laboratory conditions.

Seasonal variation is also apparent. The egg-sets of the 61 females studied during August have an average cleavage time of 46.6 minutes, while that of the 27 egg-sets studied in September is 50.4 minutes—a fluctuation of about 8 per cent.

The effects of changes in temperature upon time of cleavage need no comment. Under ordinary conditions at the Marine Biological Laboratory, the temperature varies from about 18° to 25° C. during the *Arbacia* season. Cleavage time within this range varies from 40 to 68 minutes (Loeb and Wasteneys, 1911)—a difference of more than 50 per cent. Harvey (1932, pp. 149–150) has summarized the results of other studies of temperature effects.

THE APPLICABILITY OF THE PRESENT DATA TO STUDIES OF ARBACIA EGGS GENERALLY

Since *Arbacia* eggs vary so greatly in cleavage time under various conditions, the usefulness of any time schedule of mitotic events will depend upon whether or not there is any constant relationship between the time when each mitotic phase occurs and the cleavage time.

That there is such a constant relationship is shown by the data of Table III, which presents the mitotic time schedules of the first-cleavage figure in five egg-sets, in which the experimental conditions differ as to year, time of season (July 6 to September 20), and temperature (from 15° to 25° C.). Regardless of the length of the cleavage cycle in these sets, the time when each mitotic phase occurs is the same percentage of the cleavage time. The minor deviations are random in nature and due to experimental errors.

Not only do the various mitotic phases of the first-cleavage figure in the present study occur at the same, or practically the same, percentages of cleavage time as the four other studies reported in Table III, but in the case of three of those studies the data available (but not reported) show a similar constant relationship between the phases of the sperm-aster and of the second-cleavage figure. Hence the data of the present study can be applied to *Arbacia* eggs generally. The various mitotic phases occur at the following percentages of cleavage time:

Pronuclei and Sperm-aster

Early (Fig. 1)	7 per cent of cleavage time
Middle (Fig. 2)	17 per cent
Late (Figs. 3 and 4)	43 per cent

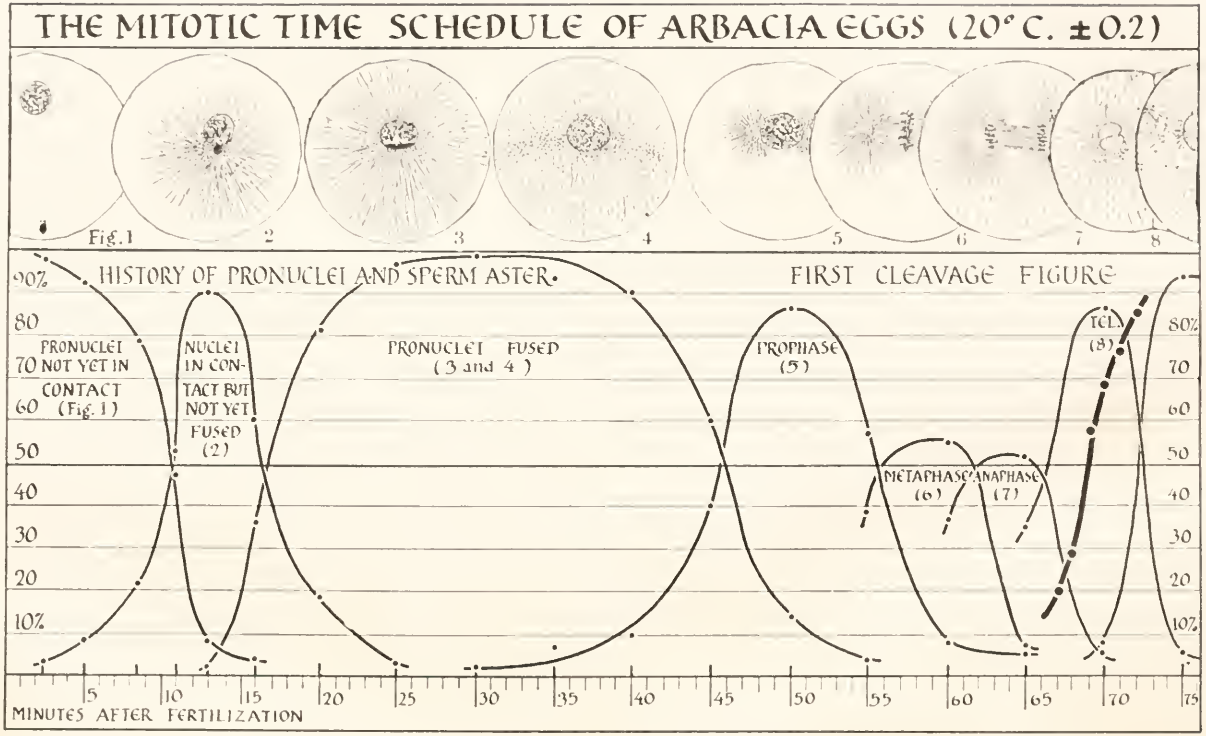
First-Cleavage Figure

Prophase (Fig. 5)	72 per cent
Metaphase (Fig. 6)	85 per cent
Anaphase (Fig. 7)	93 per cent
Fifty per cent first cleavage	100 per cent
Telophase (Fig. 8)	101 per cent
Resting (Fig. 9)	112 per cent

Second-Cleavage Figure

Prophase (Fig. 10)	136 per cent
Metaphase (Fig. 11)	147 per cent
Anaphase (Fig. 12)	152 per cent
Fifty per cent second cleavage	155 per cent
Telophase (Fig. 13)	161 per cent

Chart 1


THE MITOTIC TIME SCHEDULE OF ARBACIA EGGS (20° C. ± 0.2)
Fig. 1
2
3
4
5
6
7
8
HISTORY OF PRONUCLEI AND SPERM ASTER.
FIRST CLEAVAGE FIGURE
PRONUCLEI NOT YET IN CONTACT (Fig. 1)
NUCLEI IN CONTACT BUT NOT YET FUSED (2)
PRONUCLEI FUSED (3 and 4)
PROPHASE (5)
METAPHASE (6)
ANAPHASE (7)
TCL. (8)
90%
80
70
60
50
40
30
20
10%
80%
70
60
50
40
30
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10%
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45
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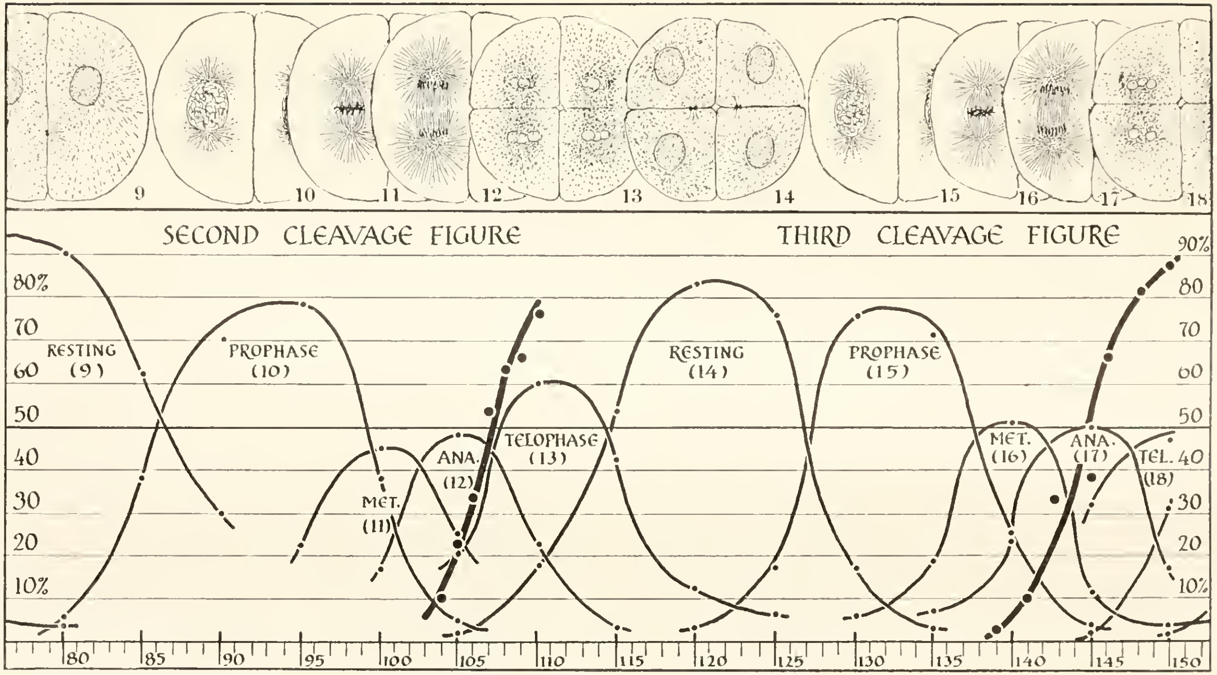
65
70
75
MINUTES AFTER FERTILIZATION



This chart presents the data of Table I in graph form. The ordinate shows the percentage of the various mitotic phases at each of the intervals reported, the latter shown on the abscissa. The three heavy lines show times of cleavages. The drawings were made in the same manner as those in a previous study (Fry and Parks, 1934, p. 477); they are enlarged 500 ×. Bouin's reagent was used, which causes a shrinkage of about one-third. The third-cleavage figures lie in a plane at right angles to that of the preceding ones, but this is not shown in the chart.

METHODS OF SECURING ARBACIA EGGS WHICH HAVE THE SAME MITOTIC TIME SCHEDULE

In many studies of *Arbacia* eggs variations in the cytological time schedule from one egg-set to another are of no significance. But where modifications in physiological factors are to be related to morphological changes, it is important to know the times of such changes as accurately as possible. If the egg-set of a single female is insufficient for an experiment, it is desirable that the several sets used have similar time schedules. Furthermore, it may be advisable that the eggs used in successive daily experiments are similar in this respect.

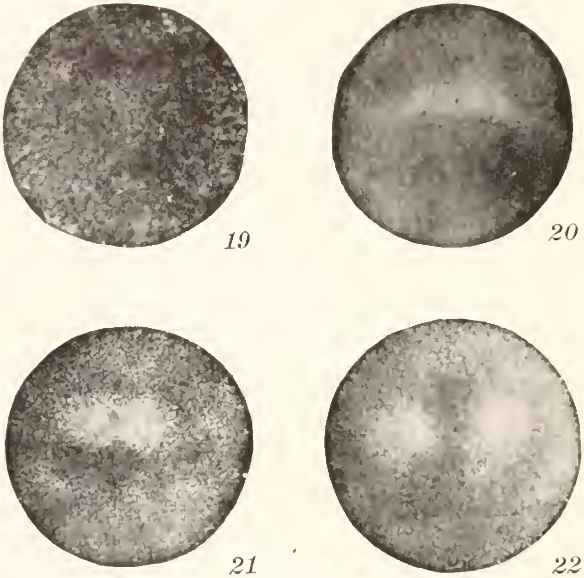

19
20
21
22

PLATE I

Photographs of the major mitotic configurations seen in living Arbacia eggs from fertilization to first cleavage.

The prints are from moving picture films made by the writer (Figs. 19 and 20) and by Professor Robert Chambers (Figs. 21 and 22), both with the aid of Mr. C. G. Grand. Figures 21 and 22 were taken under slight compression, which makes the structure more visible than it usually is. The photographs were made at a magnification of 250 ×, and are here reproduced at 460 ×.

Fig.	Mitotic stage	The same stage after fixation (Chart 1)
19	Pronuclei in contact or just fused; sperm-aster round	Figs. 2 and 3
20	Pronuclei fully fused; sperm-aster elongate	Fig. 4
21	Prophase or metaphase of the first-cleavage figure . . .	Figs. 5 and 6
22	Anaphase or telophase of the first-cleavage figure	Figs. 7 and 8

TABLE II

Variation in cleavage time among egg-sets of different females. All sets reported were in optimum condition, as shown by practically 100 per cent regular cleavage. The temperature was 23° ± 0.5. (For method of determining cleavage time see p. 98.) *Result:* On any one day there is a variation of about 10 per cent in the cleavage time of different egg-sets; between egg-sets obtained in August and in September there is an average difference of about 8 per cent.

Date 1934	Distribution of egg-sets with reference to cleavage time (i.e., time from fertilization until 50 per cent of eggs have cleaved)										
	44′	45′	46′	47′	48′	49′	50′	51′	52′	53′	Average
Aug. 4		1	1	1	1						46.5
Aug. 6			2								46.0
Aug. 7					3		1				48.5
Aug. 8	3	4	2	1	1						45.5
Aug. 10	1	1	2	1	2	1					46.6
Aug. 11	1	2	3								45.6
Aug. 12					2	1					49.0
Aug. 18	1	1	2	1	2	1					46.6
Aug. 20	2	1		1	1						45.6
Aug. 22		1	4	2	2	1					46.8
Sept. 14						2	2	3	2	2	51.0
Sept. 15					1	1	1	3	2	2	51.0
Sept. 20					2	2	1		1		49.3

TABLE III

The mitotic time schedule of Arbacia eggs during the first-cleavage cycle under various laboratory conditions. The data of egg-sets I, II, and V are from a study of the effects of temperature upon mitosis (now in progress); set III is the material of the present study; set IV is the material used in a study of viscosity and mitosis (Fry and Parks, 1934). *Result:* In spite of differences in temperature and season, and variations among egg-sets of different females, the mitotic phases always occur at the same proportional part of the cleavage time. The slight deviations are due to experimental errors.

Experimental conditions			Time schedule									
			Prophase		Metaphase		Anaphase		Cleavage		Telophase	
Egg-set	Date	Temp.	Min. after fert.	Per cent cleav. time	Min. after fert.	Per cent cleav. time	Min. after fert.	Per cent cleav. time	Min. after fert.	Per cent cleav. time	Min. after fert.	Per cent cleav. time
		°C.										
I	Aug. 4, 1934	25	30	72	35	83	38	90	42	100	43	102
II	Aug. 12, 1934	20	50	74	56	85	63	94	67	100	68	101
III	Sept. 20, 1934	20	50	72	59	85	64	93	69	100	70	101
IV	July 6, 1932	20	47	71	57	86	63	95	66	100	69	104
V	Aug. 6, 1934	15	75	67	97	86	105	93	113	100	117	103

Obviously the mixing of egg-sets with different time schedules causes a "spread" in the time when any given mitotic stage occurs.

The term *cleavage time* is used here to include the period from fertilization until 50 per cent of the eggs have divided. To determine when any one egg has divided is not a simple matter. A constriction appears first on one side; about a minute and a half later, at 24° C., it is seen on the opposite side; complete division requires another minute. In making the counts of the present study, eggs having a constriction on but one side were arbitrarily counted as uncleaved, and those having constrictions on both sides as cleaved.

In order to secure different egg-sets [1] which have the same cleavage time, preliminary tests are required. The egg-set of each female is kept separate; samples of each are fertilized simultaneously, and kept at the same temperature. When the time of cleavage approaches, the samples are observed in rapid rotation. Since one observation requires about 15 seconds, it is not advisable to work with more than eight or ten samples at a time. If larger numbers of females must be tested, samples are fertilized in groups at about 15-minute intervals. When observing cleavage, accuracy is increased if the eggs are gently stirred with a glass needle, so that they can be seen from several positions. The sets which have the same cleavage-time are then mixed and used for the experiment.[2]

When such preliminary tests are made, the eggs to be used later in the experiment are kept standing for somewhat over an hour. This, however, does not modify either the percentage of normal cleavage or the time when it occurs. Several experiments were carried out to determine the effects of such standing upon the eggs, and it was found that cleavage is unmodified for at least six hours.

This preliminary test is especially valuable when working with *Arbacia* eggs late in the season. Those familiar with this material rightfully distrust experiments carried out toward the end of the season, when the eggs of many females are in poor condition. Since it is the usual practice to mix the egg-sets of a number of females, the inclusion of subnormal sets introduces doubtful factors into the experiment. By preliminary tests, however, one can select the sets which are in optimum condition, as judged by capacity to lift distinct fertilization membranes, to cleave regularly in practically 100 per cent of the cases, and to develop into top-swimming plutei.

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[1] Methods for obtaining eggs and fertilizing them have been described by Just (1928). Additional details will be found in the writer's discussion of *Arbacia* eggs in the forthcoming *Compendium of Culture Methods for Invertebrate Animals.*

[2] The procedure described is sufficiently accurate for most purposes; if greater accuracy is necessary, the following method is generally employed. In any given sample, as soon as a small percentage of the eggs have cleaved, some of them are fixed in a 0.5 per cent solution of formol in sea-water; this is repeated at intervals until the majority have divided. Eggs thus fixed have practically the same appearance as when alive, and accurate counts can then be made.

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THE EFFECT OF WHITE LIGHT UPON THE RATE OF DEVELOPMENT OF THE RHIZOID PROTUBERANCE AND THE FIRST CELL DIVISION IN FUCUS FURCATUS[1]

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It has been observed by several investigators that the site of germination and the plane of the first cell division in the egg of the brown alga, *Fucus*, are determined by the direction of a beam of light. The rhizoid develops on the side of the egg which is least illuminated, provided, as Hurd (1920) and Kniep (1907) and others have observed, the individual eggs are at least several egg diameters apart. If egg cells lie more closely together they exert certain mutual influences upon the developmental axes which overcome the light effect. Hurd (1920) also found that the most effective part of the visible spectrum is in the blue range.

The present paper gives the results of experiments designed to measure the effect of light upon the rate of the early developmental changes in *Fucus* when eggs are spaced far enough apart to avoid mutual effects, so that the axes of differentiation are determined by the direction of illumination. Since these experiments were carried out, a search of the literature has shown that Kniep (1907) long ago noted in passing a slight accelerating effect of light on the germination of *F. vesiculosus* and *F. serratus* from the Norwegian Coast. His observation is confirmed and extended in this investigation on *F. furcatus*. A considerably more marked effect is also found.

MATERIAL

In the year 1934–35 *Fucus* has been collected at least once a week from early October until the middle of June at Moss Beach, about twenty miles south of the Golden Gate on the California coast. Two similar but distinct types of *Fucus* were found here in fair abundance, in slightly different habitats. On a large tidal shelf, a shorter type has narrower fronds and receptacles, while on a reef a larger more robust type which grows to greater length has relatively wider fronds and receptacles. *Fucus* is apparently plastic in regard to growth forms. To avoid if possible the question of growth forms and genetic forms,

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[1] This work has been supported in part by funds granted by the Rockefeller Foundation.

collection has been confined to what is believed to be one uniform type (the longer wider type) taken from the reef. A typical sample, collected early in June, is shown in Fig. 1. From Gardner's (1922) descriptions and classification, this could not be other than *Fucus furcatus*. Professor N. H. Gardner has been so kind as to identify living samples of the material which was used as *F. furcatus* f. *luxurians*, and of the smaller narrower type which was distinguished and avoided as *F. furcatus* f. *typicus*.

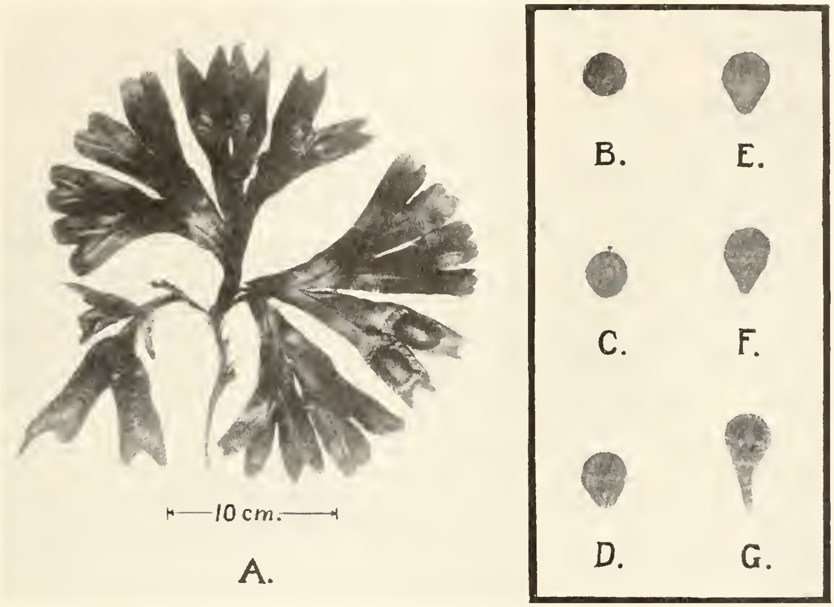

Fig. 1. (*A*) Photograph of a typical sample of the type of *Fucus* used, which has been identified as *F. furcatus* f. *luxurians* (collected early in June). The scale applies only to (*A*). (*B*)–(*G*) Developmental stages showing the development of the rhizoid protuberance and cell and (*G*) a later stage. A large egg of this form is approximately 90 microns in diameter.

Throughout the period referred to, mature gametes in excellent condition have been obtained. In the winter months new fruiting tips began to grow, and these fruited in late March, yielding larger numbers of gametes than the older tips, which, however, did not cease to bear if at all at least until well after the new tips were ripe. The new tips continued steadily to come more fully into fruit throughout the spring until and including the middle of June when collection was discontinued at Moss Beach. The present experiments were carried out on material collected in January and February.

Method

After some experimentation, an effective standard procedure was devised for caring for the material. Fruiting tips were cut off with uncontaminated scissors and placed in a gallon thermos jug which had been pre-cooled with ice. A corked bottle of ice was put in on the tips and the jug was sealed. The tips were thus kept cold and moist, but not in sea water. The jug was kept in an electric refrigerator at 4° C. as the working source of supply. Sea water was collected at the same time and after being twice passed through coarse filter paper was stored at 8° C. Fresh gametes were obtained by first washing a number of tips thoroughly in 8° C. sea water, to remove debris and previously shed gametes, and then placing them in a dish of sea water at 15° C. This served as a stimulus to shedding, and in about twenty minutes in the spring (40 minutes in the fall) eggs and sperm capsules were shed. After the tips had been in the refrigerator for three days, large numbers of gametes were obtainable, while before this time often few or none would exude. The material is good at least for well over a week when kept at 4° C., although none was used which was more than a week old.

This form is hermaphroditic, as are all the Fucaceæ on the California coast. When it was necessary to know the approximate time of fertilization, as in the present instance, a group of recently shed capsules were transferred to another dish of sea water at 8° C., where the time at which 50 per cent of the egg-bearing capsules had broken down was noted.[2] Eight eggs occur in a capsule which dissolves in sea water and ruptures, when fertilization ensues if antherozoids are present. Very small capsules containing many antherozoids are shed along with the egg capsules and many of these rupture before the egg capsules do so that motile antherozoids, or sperm, are ordinarily abundant about and on the egg capsules when these break down. In the case of an isolated capsule the time of fertilization of the eight eggs can be determined quite precisely. In the populations of capsules of nearly equivalent age in sea water, the time between rupture of the first and last capsule was usually about 15 minutes, so that the midpoint represented the time of fertilization of all of the eggs within approximately ± 8 minutes.

After capsule breakdown, the eggs were kept in a dark constant temperature room in which the air temperature was $14\frac{3}{4} \pm \frac{1}{4}$° C.

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[2] It was not entirely necessary to have the capsules break down at 8° C. instead of at 15° C., but uniformly single eggs emerge at cool temperatures, while as the temperature increases, so does the proportion of eggs which fuse to form polyploid giants (Whitaker, 1931).

The heat capacity of the water about the eggs reduced the temperature variation considerably. The eggs were in the dark except for brief exposures to dim red light, or during experimental illumination.

Following fertilization, the *Fucus* egg secretes a gelatinous substance which gradually becomes sticky and finally hardens to form a cellulose wall (Whitaker, 1931) which attaches the egg quite firmly to such substrates as glass or agar jel. In manipulating *F. furcatus* eggs at 15° C., the stickiness becomes conspicuous about 45 minutes after fertilization, and is very pronounced at 2 hours. The manipulation, transfer, and placing of eggs was completed in these experiments within 20 minutes after fertilization.

A large double compartmented box was constructed inside the constant temperature room, painted black inside and out. It was so arranged that a light bulb, in fixed position at one meter distance, laterally illuminated a levelled platform in one compartment, while a similar platform in the other compartment remained in complete darkness. An electric fan was so arranged, in conjunction with baffles and slits which admitted no light, that copious and equal volumes of air passed continually from the constant temperature room through the experimental chambers and out behind the light. Calibrations under working conditions showed that the two chambers were at the same temperature, and at the same temperature as the constant temperature room, namely 14¾ ± ¼° C. The white light source used in the experiments was a General Electric frosted 40-watt bulb, operated on 110 volts, A.C. With facilities and assistance kindly provided by Dr. Arthur Giese, this was calibrated by means of a thermopyle against a standard light. The intensity under working conditions at one meter distance from the bulb was such as to give total energy of approximately 1500 ergs per second per cm.^{2}.

Measuring Time Lapse

Soon after fertilization, eggs were thinly scattered in sea water over the bottoms of square Petri dishes (8.5 cm.^{2}) which presented flat surfaces to the laterally transmitted light, and also served as moist chambers. In any given experiment, the eggs reared in the light and in the dark were arranged by equipartition and random dispersion to be of equal concentration and statistical proximity on the bottom of the dish, as well as to be of equal concentration per unit volume of sea water. In the different experiments the same conditions in these regards were approximated. The average concentration of eggs was of the order of 25 eggs per cm.^{2} of bottom, and 60 eggs per cc. sea water. The eggs are about 65–90 microns in diameter. In recording results all eggs were ignored which were within 5 egg diameters of another egg, because of certain mutual effects of these eggs when they lie within two or three egg diameters of neighbors. The great majority of the eggs were much more than 5 egg diameters apart.

In Experiments 9 and 10 (Fig. 2) duplicates were run in isosmotic 1 per cent, and in Experiment 9 also in 2 per cent, dextrose. Solutions of dextrose in triple distilled water were made approximately isosmotic with sea water (11/12 M). Enough of this was added to sea water to give a resulting solution containing one, or in the 2 per cent two, grams of dextrose per 100 cc.

Within 20 minutes after fertilization one set of dishes bearing eggs was placed in the illuminated chamber of the dark box, and another set in the dark chamber. After preliminary survey, two arbitrarily chosen criteria of developmental stage were selected which could be quite sharply identified. The first of these was the early but unmistakable protuberance at one side of the egg, which is to give rise by extension and cell division to the rhizoid cell. The first cell division in *Fucus* results in two morphologically different cells which ordinarily lie side by side in the plane of the substrate (Fig. 1) and which by further division give rise respectively to the thallus and the rhizoid of the new plant. The developmental axis is thus, in the normal course, determined by the time the rhizoid protuberance appears, and is indicated by it. The second end point selected was the development of the cell plate to an arbitrarily chosen stage which was distinctly and sharply distinguishable, and which marked the beginning of the 2-cell stage. In the absence of cytological study, it is not certain precisely to what extent the cell plate had divided at this time.

The chief measure sought was the time after fertilization, in the dark and in the light, when 50 per cent of the population had reached (or passed) the stages in question. In the first seven experiments more numerous counts were made principally just before, during, and just after this time had been reached. It was then found to be both more convenient and more accurate, as well as to involve less disturbance of the dishes, to make percentage counts at less frequent intervals and to interpolate the 50 per cent point on the sigmoid curve which could be drawn through the points. This method was followed in Experiments 8, 9 and 10, the results of which are shown in Fig. 2. The eggs reared in the dark were observed and tabulated with red light, the eggs reared in the light with white light. Checks showed that the different lights used made no difference in identifying the stages. The Petri dish was moved back and forth across the stage of the microscope so that eggs were observed (with low power) as one might read a printed page. Each count involved from 100 to 300 eggs.

Results and Discussion

As may be seen in Table I, the time lapse from fertilization until 50 per cent of the population had attained (or passed) the two stages in question varied somewhat in the different experiments, but in all cases the rate of development was decidedly more rapid in the light than in the dark. Various factors which may affect the rate were naturally more uniform among the experimental groups of a given experiment than among different experiments. Therefore the ratio of the time lapse in the light and in the dark in a given experiment is a more useful measure for comparison, and such ratios are indicated at the right in Table I.

Table I

Time in hours from fertilization until 50 per cent of a thinly spaced population of eggs at 14¾ ± ¼° C. attained or passed the early rhizoid protuberance and the early cell plate stages.

Experiment number	Number of eggs	Time in hours after fertilization				$\frac{\text{Time in dark}}{\text{Time in light}} \times 100$	
		Fifty per cent rhizoid protuberance		Fifty per cent cell plate		Rhizoid protuberance	Cell plate
		Light	Dark	Light	Dark		
1 to 7	207 av.	15.2	20.0	22.2	25.5	132	115
8	182	12.7	17.0	20.5	24.0	134	117
9	146	13.7	17.7	22.1	25.1	130	114
10	145	14.4	16.5	22.9	25.3	115	110
Average........		14.0	17.8	21.9	25.0	128	114

In deriving the final averages, Experiments 8, 9, and 10 as a group are weighted equally against Experiments 1–7 inclusive to arrive at what is probably the best approximation, since the former were carried out with special care and after more experience. The results of Experiments 8, 9, and 10 are shown graphically in Fig. 2, which also shows the time span of protuberance and cell plate formation in a population. It is seen (Table I) that it takes about 28 per cent more time for rhizoid protuberances to develop in the dark than in moderate white light (1500 ergs/sec./cm.^{2}), and about 14 per cent longer for cell plates to form. In absolute time, the rhizoid protuberance develops 3.8 hours sooner in the light, while the cell plate develops 3.1 hours sooner. The primary and actual acceleration in the light is therefore only in the processes leading to protuberance formation. The cell plate formation in fact loses some of the gain.

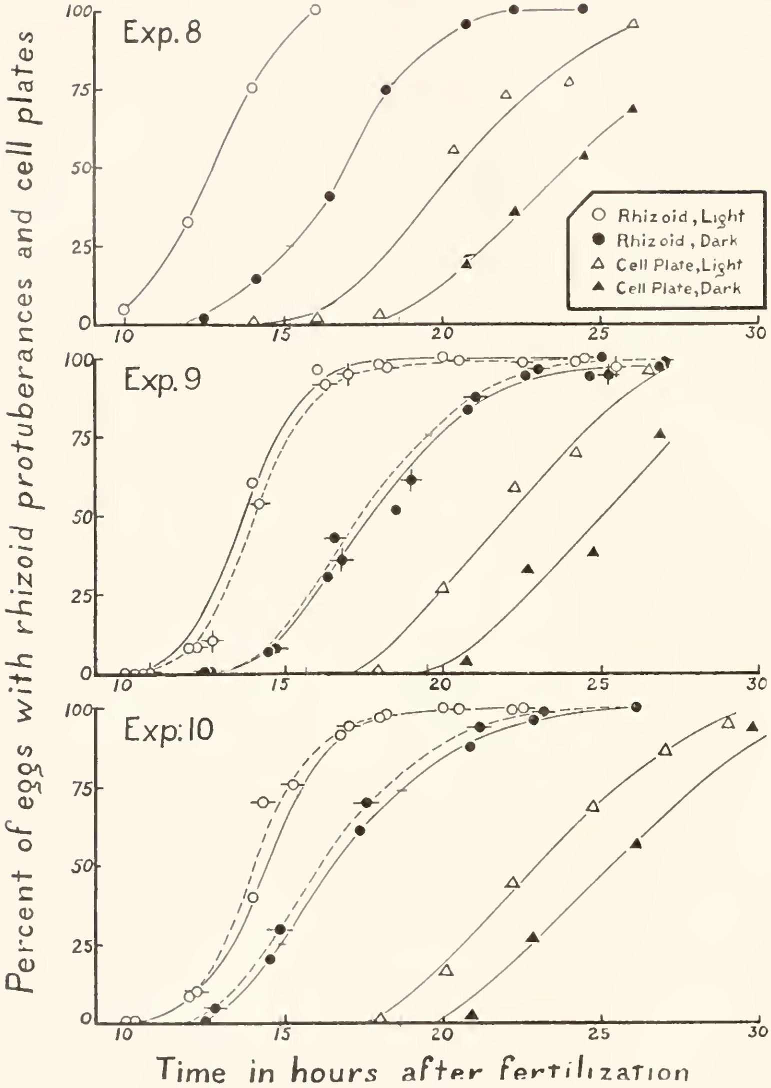

FIG. 2. Percentages of eggs in thinly spaced populations at 14¾ ± ¼° C. which attained or passed the stages indicated with passage of time after fertilization. In Experiments 9 and 10 the rhizoid protuberance points which bear two wings and four wings, through which dashed curves have been drawn, represent runs in sea water containing 1 per cent and 2 per cent dextrose respectively.

In Experiments 9 and 10 duplicate runs both in light and dark were made which differed from the main runs only in that the sea water contained 1 per cent dextrose (isosmotic), and in Experiment 9 also 2 per cent dextrose. The time lapses to the development of rhizoid protuberances are shown in Fig. 2. These results confirm the indications of rough preliminary tests in showing that the presence of 1 per cent dextrose in the medium does not speed up the development of the rhizoid protuberance in eggs in the dark to, nor appreciably toward, the rate in eggs in the light. A slight stimulation of the eggs in the dark compared with the eggs in the light is indicated (average = 2 per cent) but this is within the limits of error. Since it is not certainly known whether dextrose can enter these eggs, nor whether it must enter to be metabolized, no positive conclusion can be drawn from the negative effect of 1 per cent dextrose. As far as these results go, however, they suggest or at least leave the way open for the view that the stimulative effect of light upon the rate of formation of the rhizoid protuberance is not primarily a nutritive phenomenon.

Summary and Conclusions

1. Data are given upon the extended breeding season of the hermaphroditic *Fucus furcatus*, and means of obtaining uniformly fertilized unfused eggs are described.

2. When thinly spaced eggs are reared in filtered sea water at 14¾ ± ¼° C., with lateral illumination by moderate white light (1500 ergs/sec./cm.^{2}), the time lapse from fertilization until 50 per cent of a population have developed early rhizoid protuberances (when the developmental axis is determined and indicated) is approximately 14.0 hours.

3. It takes approximately 28 per cent more time for the same development in the dark (at 17.8 hours).

4. Under the same conditions as in 2, 50 per cent of a population have developed cell plates, and have entered the early 2-cell stage, at approximately 21.9 hours after fertilization.

5. It takes approximately 14 per cent more time for the same development in the dark (at 25.0 hours).

6. White light thus markedly speeds up processes leading to the development of both of these structures. It accelerates the protuberance formation (involving softening of the cellulose wall on the side of the cell least illuminated) to a relatively greater extent. In absolute time, the protuberance formation is advanced 3.8 hours in the light, the cell plate 3.1 hours. The primary and actual acceleration is therefore only in the formation of the rhizoid protuberance, the cell plate formation merely holding most of the gain.

7. The presence of 1 per cent (isosmotic) dextrose in the sea water does not appreciably affect the rate of protuberance formation either in the dark or in the light.

The author is indebted to Mr. Edward Lowrance for assistance in carrying out the experiments.

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EXPERIMENTS ON LIGIA IN BERMUDA

IV. The Effects of Heavy Water and Temperature [1]

T. CUNLIFFE BARNES

(*From the Osborn Zoölogical Laboratory, Yale University*)

Three previous papers of this series (Barnes, 1932, 1934, 1935) have described salt effects and space orientation of the littoral isopod *Ligia baudiniana* in Bermuda. Since this species of *Ligia* is a sub-tropical form living under fairly uniform temperature conditions, it was decided to establish the upper temperature range in air and in sea water and to determine the effect of immersion in concentrated heavy water, which is equivalent to a drop in temperature from the standpoint of energy content (*cf.* Eyring and Sherman, 1933; Barnes and Gaw, 1935; Barnes and Warren, 1935).

Methods

For the study of lethal temperatures a freshly collected adult was placed in a bottle of approximately 300 cc. capacity immersed in the water of a thermostat warmed by a knife heater controlled by a mercury relay. Fifty cc. of sea water or filter paper moistened with sea water were placed in the bottle. Air was pumped continuously through a spiral of glass tubing in the thermostat to bring it to the required temperature before it passed through the bottle. The time of death was taken as the point when the animal ceased all motion and would not respond when the bottle was violently jarred. All specimens were returned to sea water at normal temperature (27° C.), but none recovered after this end point was reached. Although the thermostat was accurate to 0.1° C., the temperature of the specimen bottle dropped 0.5 − 1° C. for a few minutes after the specimen was introduced.

For the determination of the temperature characteristics of the gill-beat, the animal was placed in 2 cc. of sea water in a test tube containing the bulb of a thermometer and a tube through which air was bubbled continuously. The test tube was immersed in a beaker of water at 27° C. and pieces of cracked ice were added with forceps to lower the temperature. Since only 2 cc. of 96 per cent heavy water were available, the experiment had to be carried out on a small scale

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[1] Contributions from the Bermuda Biological Station for Research.

(including the controls). The temperature was constant to within 0.4° C. during one reading (time for 10 beats of the pleopods).

Further Experiments with Salt Solutions

Sea water was used as the immersion medium in the temperature studies although animals will not live indefinitely in this medium. Artificial sea water in which the two most toxic ions K and Mg were reduced did not give better results than natural sea water (Table I). It will be seen from the table, however, that artificial sea water with half the normal Mg content is more favorable than artificial sea water with the normal amount of Mg. As was pointed out in a previous paper (Barnes, 1935), the K content of artificial sea water has to be increased four-fold to produce easily recognized toxic effects. Three seasons work with *Ligia* has shown the great variation that occurs in the survival times of specimens in sea water or salt mixtures similar to sea water and it is now evident that many tests have to be run with each solution, including a parallel series of sea water controls with animals collected at the same time and place, and the probable error and coefficient of variation worked out (Table I). The probable error is calculated from Peter's formula and the coefficient of variation is expressed as 100 × P. E. divided by the mean. The earlier results with toxic solutions of single salts are clear-cut and show little variation, but the great variation in ternary salt solutions can be seen in the following sample giving survival times of 14 animals each in 100 cc. of sea water: 5.5, 5.5, 5.5, 5.5, 14.5, 14.5, 14.5, 21, 29.5, 31, 31, 53.2, 192 and 240 hours respectively.

Table I

Longevity of Ligia in salt mixtures

Experiment No.	Solution *	Average longevity	Coefficients of variation	Maximum longevity	No. specimens tested
		hours		*hours*	
1	100 NaCl, 2.5 $CaCl_2$, 1 KCl, 11.6 $MgSO_4$	23± 2.3	10	96	36
	sea water control	61± 9.0	14	214.5	36
2	100 NaCl, 2.5 $CaCl_2$, 1 KCl, 5.8 $MgSO_4$	40± 4.0	10.3	288	63
	sea water control	52± 6.4	12.2	336	63
3	100 NaCl, 2.5 $CaCl_2$, 1 KCl	20± 2.5	12.4	89.5	32
	sea water control	82±12.4	15	288	32
4	100 sea water + 1 KCl	39± 7.8	20	288	17
	sea water control	57±11	19.2	216	16

* The numbers indicate cc. of 5/8 M solutions.

SURVIVAL IN AIR AND IN WATER AT HIGH TEMPERATURES

Ligia baudiniana dies in about one hour if placed in aerated sea water at 38° C. (Table II), but at this temperature in air (over filter paper moistened with sea water) it survives so long that the longevity could not be determined. At 42° C., however, specimens in air die in slightly less than an hour and 50° C. is rapidly fatal in both water and air. If a specimen is slowly heated in 50 cc. of sea water with a temperature rise of about $\frac{1}{2}$° C. per minute, the gill-beat becomes irregular at 39 – 40° C. and death occurs at 40–42° C. The Arrhenius equation, velocity $\alpha e^{-\mu/RT}$, was applied to the data for the rate of killing in air and yields a value of μ, the temperature characteristic, of 56,400 calories.

TABLE II

Survival times in sea water and in air at high temperatures

Sea water			Air		
Temperature	Average survival	No. specimens tested	Temperature	Average survival	No. specimens tested
° C.	*minutes*		*° C.*	*minutes*	
38	61.8 ± 4.7	15	42	52.7 ± 5.7	14
44	7.6 ± 1	8	44	20 ± 2	19
50	0	8	45	11.5 ± 3	6
			50	3 ± 0.3	16

TEMPERATURE CHARACTERISTICS FOR THE GILL-BEAT IN "HEAVY" SEA WATER

The flapping of the pleopods is regular over a temperature range of about 10–38° C., but the frequency is too great above 28° for accurate timing with a stop-watch. In a typical specimen the time for ten beats increases from 2.5 seconds at 26° to 7 seconds at 10°. The gill-beat begins to be irregular at 10° and stops below this critical temperature. After exposure to 10° or below the frequency remains lower than normal when the temperature is raised (Fig. 1). Therefore, the data used in calculating the temperature characteristic were obtained from readings taken as the animal was cooled from 27° to 10°. As will be seen from Fig. 1, the readings on the upward temperature curve are all lower than those in the downward direction. For the heavy water experiments 2 cc. of sea water were evaporated down and the salts redissolved in 2 cc. of 96 per cent D_2O (specific gravity 1.1037). The same procedure was used for the controls except that the salts from the evaporated sea water were taken up in ordinary distilled water. The frequency of the gill-beat immediately falls in "heavy" sea water. Thus at 20° C. a typical determination for the time for ten beats was 3.8 seconds in H_2O and 4.5 seconds in D_2O. In Fig. 1 it will be seen that this lower frequency obtains throughout the entire temperature range in heavy water and that exposure to 10° is followed by a further reduction in rate as in ordinary sea water.

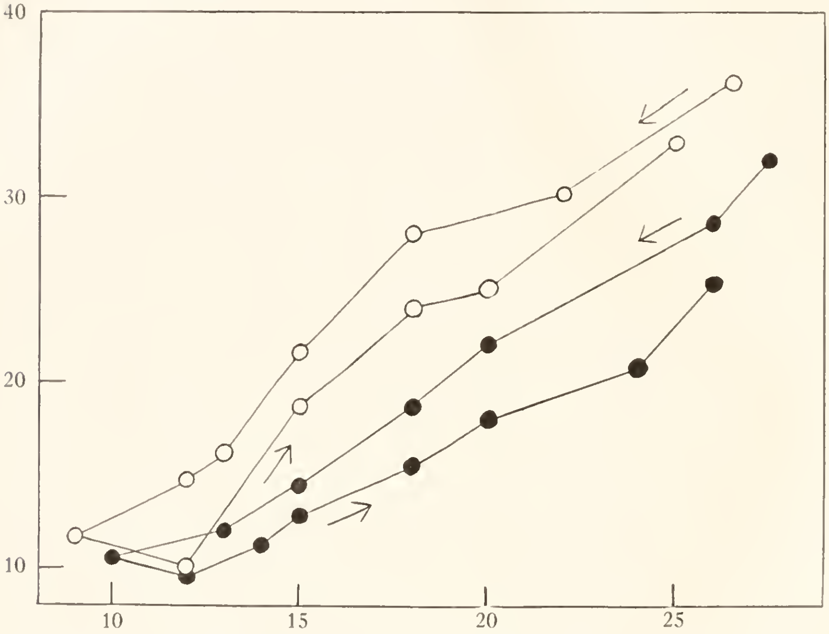

FIG. 1. Rate of beating of the pleopods of *Ligia* as a function of temperature. Ordinates, rate expressed as 100 divided by the time for 10 beats. Abscissæ, temperature in degrees centigrade. Open circles represent a specimen in ordinary sea water. Black dots represent a specimen in sea water containing 96 per cent heavy water. The rate was taken at the higher temperature first and then at progressively lower temperatures to about 10° where the beat becomes irregular. Then the temperature was raised, as indicated by the arrows. Each point is the average of five determinations of the time for 10 beats. Each run from 28° to 10° or 10° to 28° required about 40 minutes.

The data for both ordinary and "heavy" sea water are plotted in Fig. 2 according to the Arrhenius equation

$$ln \frac{R^2}{R^1} = \frac{\mu}{2}\left(\frac{1}{T^1} - \frac{1}{T^2}\right),$$

where R^1 is the rate at T^1 and R^2 is the rate at T^2. It appears that the slope of the line changes sharply at 16.5° C. in H_2O sea water and at 19.5° C. in D_2O sea water. The frequency is greatly reduced in heavy water but it is interesting to observe that the lines for both media are parallel. In ordinary sea water the temperature characteristics are: 16,000 calories for the interval 10–16.5° C. and 8,400 calories between 16.5 and 27° C. For "heavy" sea water the μ values are; 15,000 calories, 10–19.5° C. and 8,400 calories between 19.5° and 27° C. The significance of these findings will be discussed in the sequel. There were no signs of injury during the hour and a half run in heavy water and the reduction in frequency of the gill-beat was reversible after return to ordinary sea water. Thus a specimen in which the time for ten beats was 5.5 seconds at 17° in D_2O showed a typical rate of ten beats in 4.2 seconds at 17° after return to sea water.

It will be noted in Fig. 2 that the scatter of the plotted points is much greater in H_2O sea water than in D_2O sea water. Owing to the scarcity of concentrated heavy water, the determinations were made from two specimens. Although many animals were used in ordinary sea water, the data from only two typical specimens are plotted in Fig. 2 as a basis for comparison with the D_2O results. This procedure is

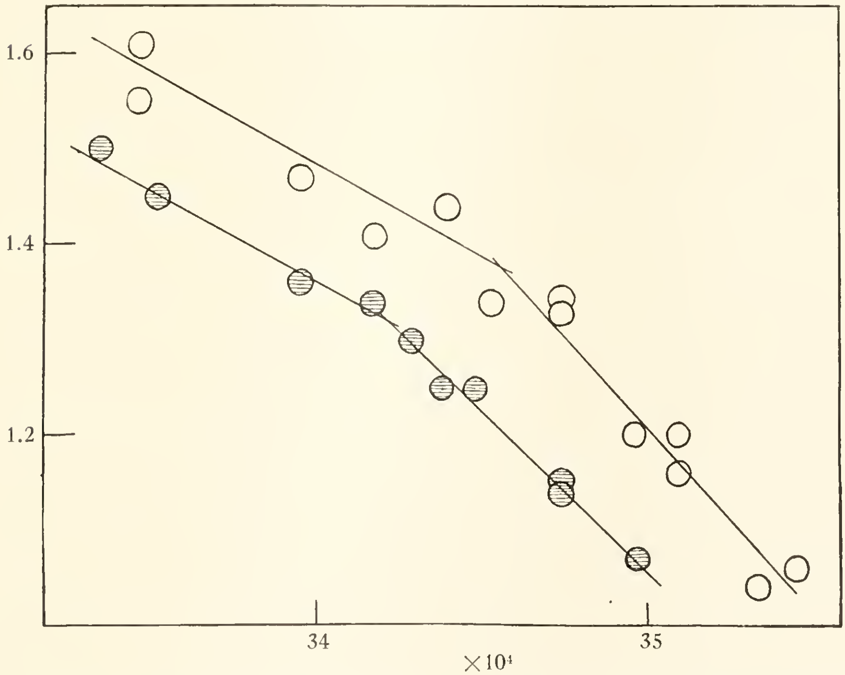

FIG. 2. Relation between the log of the rate of gill-beat in *Ligia* (ordinates) and the reciprocal of the absolute temperature (abscissæ). Open circles represent data from two specimens in ordinary sea water. Shaded circles represent readings from two individuals in sea water containing 96 per cent heavy water. In the upper graph the lines intersect at 16.5° C., in the lower at 19.5° C. (See text.)

justified by the fact that in ordinary sea water the temperature characteristics are the same as those obtained by Numanoi (1935), who used hundreds of specimens of the closely allied *Ligia exotica*. The specimens used in the 96 per cent D_2O measured 13 and 14 mm. in length (not counting uropods and spines) which were large in comparison to the 2 cc. of water, so that the concentration in the tissues was probably about 80 per cent heavy water.

Discussion

Evidence is accumulating that the inability of *Ligia* to survive when immersed in sea water is not entirely determined by the relative proportions of the chief cations. However, it is interesting to note that a reduction by half of the Mg content of artificial sea water permits greater longevity in that medium but a synthetic sea water of greater sustaining power than natural sea water has not yet been found.

The studies of the rate of killing of the isopod at high temperatures indicate an abrupt transition from the rate of killing curve to the ordinary curve of life duration as found by Brown and Crozier (1927) in cladocerans. The lethal temperatures for *Ligia* in Bermuda do not appear to be higher than those for organisms in the temperate zone, but the minimum temperature for the gill-beat, 10° C., is four degrees higher than that of *Ligia exotica* in Japan (Numanoi, 1933). The high temperature characteristic for the rate of killing of *Ligia* at high temperatures is similar to values obtained from such processes as the coagulation of proteins by heat (Buchanan and Fulmer, 1930, p. 52). The more rapid lethal effect of high temperature in water than in air is a common phenomenon and probably not connected with the greater longevity of *Ligia* in air at ordinary temperatures (25–28°).

The temperature characteristic of 16,000 calories obtained for the gill-beat is one repeatedly encountered for respiratory processes (Numanoi, 1933) and is associated with the catalysis of oxidation by iron (Crozier, 1924). The μ values obtained by Numanoi (1933) for the gill-beat of *Ligia exotica* in Japan, i.e., 15,200–17,000 calories between 6–16° C. and 7,000–8,200 calories between 16 and 30° C., are precisely the same for *Ligia baudiniana* in Bermuda. These two values occur together frequently for respiratory processes.

The application of the Arrhenius equation to the data for the gill-beat in heavy water should throw light both on the mechanism of action of the heavy hydrogen isotope and on the validity of the Arrhenius equation in biological systems. Crozier (1935) has recently discussed modifications of temperature characteristics from which it is clear that if the several discrete magnitudes of μ persist in spite of modifications of the temperature graph, it may be concluded that the temperature characteristic represents the molecular energy of activity of a specific chemical process. Now it will be seen in Fig. 1 that in spite of the great reduction in frequency by heavy water, the rectilinear relation is not destroyed and the same discrete values of μ can be recognized as for the process in ordinary water. Not only so, but the data from the heavy water series fit the equation even more closely in that the points all lie on the line.

It appears that the heavy water reduces the frequency of the gill-beat without changing the fundamental regulating mechanism. A similar effect is seen in the action of adrenalin on the heart (Crozier, 1926), in which the frequency is greatly increased but the temperature characteristic is not changed. Urey and Teal (1935), when discussing the energies of activation of chemical reactions in heavy water, point out that no difference in the activation energies need be expected in reactions in which hydrogen is not directly involved. On the other hand, the reaction between deuterium and bromine to form DBr has an activation energy of 19,870 calories which is 2,100 calories higher than that for the reaction $H + Br = HBr$, the difference being about equal to the difference in the zero point energy of the two hydrogen isotopes.

It remains to be seen if there are biological processes in which the temperature characteristic is changed greatly in heavy water. The rate of pulsation of the contractile vacuole in *Paramecium* (Barnes and Gaw, 1935) has three values of μ over the temperature range in ordinary water i.e., below 16°, 24,000; 16–22°, 17,000; and above 22, 14,000; but in heavy water the value is 22,000 over the entire range. The rate of pulsation of the turtle's auricles (unpublished data), like the gill-beat in *Ligia*, yields a μ value of 16,000 in both D_2O and H_2O. It is hoped that further temperature studies in heavy water will afford new and significant information concerning the regulation of physiological processes.

The effect of heavy water on the rate of the gill-beat is equivalent to a drop in temperature of 3–4° C. This is also seen in the critical temperature or point in the graph (Fig. 2) where the slope of the line changes. The line breaks at a point 3° higher in the plot for the heavy water experiment. It is proposed that the effect of heavy water on a physiological process should be expressed as an "*equivalent drop in temperature*" of a specific number of degrees. This is similar to a new method of expressing the configuration of the water molecules in a dilute solution of strong electrolytes proposed by Bernal and Fowler (1933)—the "structural temperature" or that temperature at which pure water would have effectively the same inner structure as the given solution.

SUMMARY

1. The survival of *Ligia* in artificial sea water is doubled if the Mg content is reduced by one half.

2. In spite of the fact that *Ligia* cannot survive long periods in natural sea water, no artificial sea water has been found to be a more favorable medium.

3. Exposure of about one hour to sea water at 38° C. or to moist air at 42° C. is fatal to *Ligia*.

4. In natural sea water the beating of the pleopods of *Ligia baudiniana* yields temperature characteristics of 16,000 calories between 10 and 16° C. and 8,400 calories between 16 and 28° C. These are typical of respiratory processes.

5. The reduction in the frequency of the gill-beat in sea water containing 96 per cent heavy water is equivalent to that produced by a drop in temperature of about 3–4° C. Similarly the critical temperature in heavy water is shifted three degrees higher.

6. In sea water containing 96 per cent heavy water the gill-beat yields temperature characteristics of 15,000 calories between 10 and 19 and 8,400 calories between 19 and 28° C.

7. It is concluded that the lower energy content of heavy water slows down the beating of the gills but does not change the fundamental regulatory mechanism.

It is a pleasure to express my gratitude to Dr. J. F. G. Wheeler, who placed the facilities of the Bermuda Station at my disposal.

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THE BIOLOGY OF CALANUS FINMARCHICUS IN THE GULF OF MAINE AND BAY OF FUNDY

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INTRODUCTION AND METHODS

The problem considered in the present paper concerns the production and dispersal of *Calanus finmarchicus* in a partially enclosed body of water. There have been two principal objectives; first, a determination of the breeding seasons, annual number of broods, rate of growth and length of life; second, an evaluation of different spawning areas, and dispersal of eggs and larvæ.

Monthly cruises were made from July 28, 1931 to September 29, 1932 in the Bay of Fundy and from April to September, 1932, in the inner part of the Gulf of Maine, with one cruise in the outer Gulf and along the south coast of Nova Scotia, yielding in all a total of 838 zoöplankton samples (Fig. 1). In August 1931 one line of stations was taken in the Gulf.[2]

For convenience in description, the region has been subdivided into four areas shown in Fig. 1. For the sampling of eggs and immature stages, the pump was used during the summer, a volume of 250 liters of water from each of four levels, 0, 10, 30, and 50 meters being filtered at each station. Analyses were made by the Sedgewick-Rafter counting method, and the number of individuals per cubic meter calculated. In determining horizontal distribution, in the present report, the value taken for each station represents the mean of the four levels. Until weather conditions permitted the use of the pump (end of June), half-meter nets (two meters in length) of No. 10 silk were utilized. The hauls were of 15 minutes duration and were oblique from 50 meters to the surface, the distance being divided into six steps at intervals of 10 meters each. The counts were made by the same method used in the pump samples, but the number of organisms per minute of towing was taken as the unit of measure.

IMPORTANCE

Calanus finmarchicus is the most abundant animal in the Gulf of Maine, occurring offshore throughout the year and well into inshore waters except during the warm summer months when its distribution is somewhat variable. In Frenchmans Bay, with temperatures as low as 7–9° C. persisting in the lower levels, it was found abundant throughout the summers of 1929, 1930, and 1932, but in shallower or more turbulent estuarine areas such as Passamaquoddy Bay, where the whole water mass responds more rapidly to seasonal warming (10–12° C. after August 1), *Calanus* was comparatively sparse between June and October.

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[1] Contribution No. 85.

[2] Investigations for the International Passamaquoddy Fisheries Commission.

FIGURE I

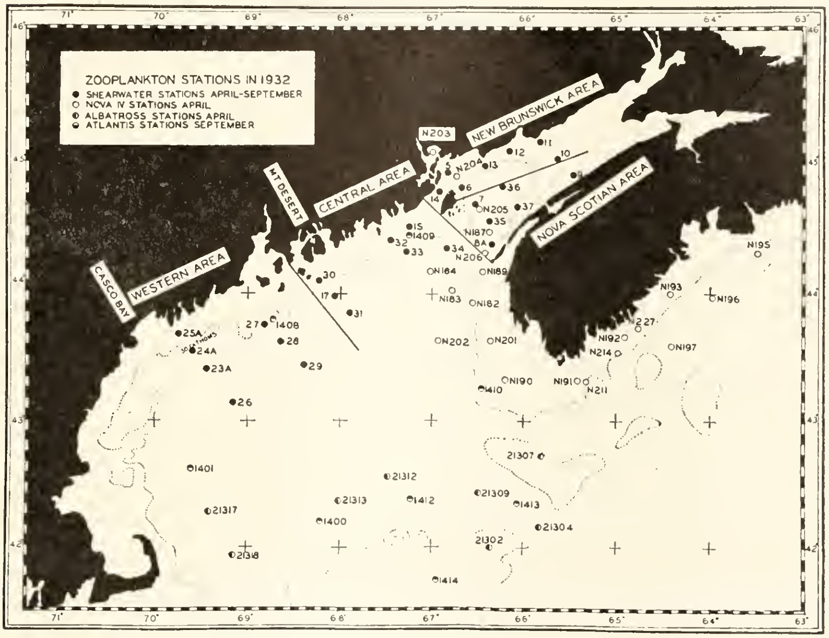
ZOOPLANKTON STATIONS IN 1932
● SHEARWATER STATIONS APRIL-SEPTEMBER
○ NOVA IV STATIONS APRIL
◐ ALBATROSS STATIONS APRIL
◒ ATLANTIS STATIONS SEPTEMBER
71°
70°
69°
68°
67°
66°
65°
64°
63°
46°
45°
44°
43°
42°
N203
NEW BRUNSWICK AREA
CENTRAL AREA
MT DESERT
NOVA SCOTIAN AREA
WESTERN AREA
CASCO BAY
11
12
10
9
5
N204
13
6
36
14
7
N205
37
35
15
1409
N187
32
33
34
8A
N206
N195
30
17
ON164
ON189
N183
ON182
N193
ON196
31
27
1408
25A
50 FATHOMS
24A
28
23A
29
ON202
ON201
227
N192
N214
ON197
ON190
1410
N191
N211
26
21307
1401
21312
21313
1412
21309
1413
21317
1400
21304
21302
21318
1414

FIG. 1. Zoöplankton stations in 1932.

Its importance in the region is indicated in Tables I and II giving the relative percentage by number of *C. finmarchicus* to the total zoöplankton population. Within the Bay of Fundy, *Calanus* was found most abundant at all times in the vicinity of the deep basin, with year values increasing upon approaching the entrance. Thus, as indicated below, at Station 5 in the Quoddy region the mean for the year was 32.4 per cent, at Station 6 east of Grand Manan 37 per cent, and at Station 8A near the mouth on the Nova Scotian side, 41.8 per cent.

In the Gulf of Maine the highest values occurred in the central area where production of many of the pelagic species breeding during the summer appears to be largely unsuccessful. Here *C. finmarchicus* ranged from 40 per cent to 90 per cent, except in April when locally spawned larvæ of *Balanus balanoides* dominated the hauls.

Bigelow (1926, p. 199) found that in the Gulf of Maine *Calanus* averaged approximately 55–60 per cent of the total copepod content. In 1932 it averaged 60.4 per cent of the zoöplankton population in May with a total copepod content of 77.2 per cent and 39.9 per cent for the period April to September. In the eastern Atlantic Ruud's (1929, p. 11) records indicate that off Möre in 1926 and 1927 *C. finmarchicus* formed about 13–15 per cent of the copepod population in March and rose to 63.4 per cent following vernal augmentation. The average for the period March–July during the two years was approximately 39 per cent.

Table I

Relative percentage by number of *C. finmarchicus* to the total zoöplankton population, 1931–32, Bay of Fundy.

1931–32	Bay of Fundy			
Month	Mean	Station 5	Station 6	Station 8A
September	28.5	17.5	6.7	61.4
October	23.4	23.4	—	—
November	31.3	36.1	34.6	23.3
December	37.9	61.2	34.2	18.2
January	25.9	25.9	—	—
March	61.6	73.2	57.3	54.4
April	34.8	49.7	36.6	18.1
May	41.0	4.8	56.7	61.5
June	36.4	15.5	42.5	51.2
July	29.0	29.0	—	—
August	31.4	20.0	27.8	46.3
Mean for year	35.5	32.4	37.0	41.8

It would thus appear that in the Gulf of Maine *C. finmarchicus* forms a more important member of the zoöplankton population than in the region off Möre, although at no time were eggs found in such quantities as reported from certain areas between Norway and Iceland, where Damas (1905, p. 12) found them at times comprising practically the entire haul. In the Gulf of Maine at the peak of June spawning (1932) eggs of *Calanus* were the dominant form, but were everywhere outnumbered by the combined eggs and larvæ of other species. In the Bay of Fundy, also, Willey has commented (1921, p. 189) on the absence of *Calanus* eggs in any such relative abundance as reported by Damas. However, the importance of this species is evidenced by the manner in which the total volume of zoöplankton follows the fluctuations in the *Calanus* content (Fig. 2), a condition also noted by Ruud (1929, p. 12) for the Möre area.

Regional Distribution of the Adult Stock

The quantitative distribution of adult *C. finmarchicus* (including late copepodite stages) in the Gulf of Maine as described by Bigelow (1926, p. 188) accords so closely with present data that little need be added here. Although almost everywhere abundant, Bigelow found marked horizontal variations. Together with other boreal plankton animals the smallest numbers were found in 1931 and 1932 in the Bay of Fundy, particularly on the New Brunswick side, and along the adjacent coast of Maine to Penobscot Bay. However, although the values were uniformly low, due to equally small numbers of other boreal zoöplankton species, *C. finmarchicus* is relatively almost as

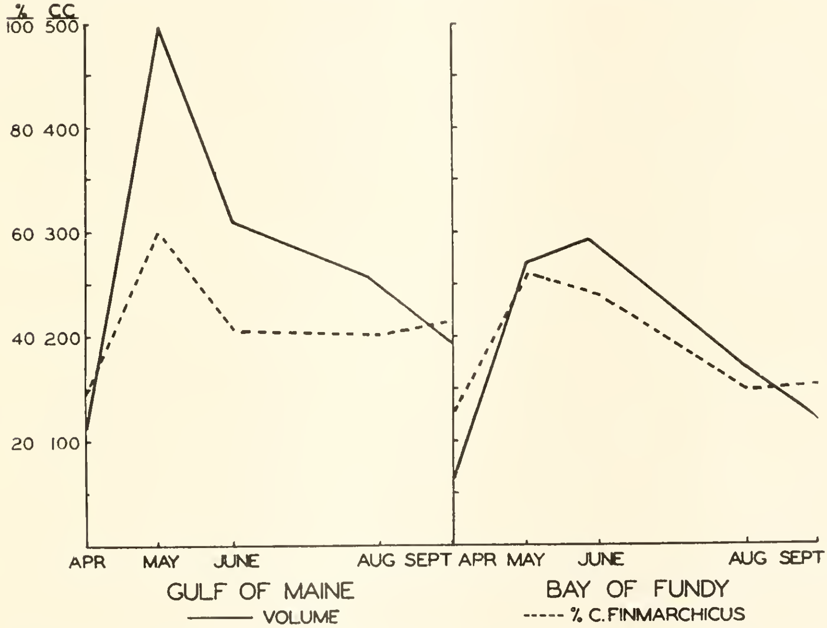

FIG. 2. Volume of zoöplankton and relative percentage by number of *C. finmarchicus* (adults and late copepodites) in the Gulf of Maine and Bay of Fundy in 1932.

important a member of the population in this region as elsewhere in the Gulf. The largest numbers in the Bay of Fundy occurred in the deep basin and along the Nova Scotian coast; in the Gulf of Maine, at the outer stations.

PRODUCTION AND DISPERSAL

Propagation of *Calanus finmarchicus* has been found to vary greatly in different localities, both in the rate of development and the number of broods. Available records indicate that in the southern portion of its range development is relatively rapid and spawning may begin as early as January. Proceeding northward into colder water, the period of vernal augmentation becomes increasingly later, development slower, and there are fewer broods.

In the Clyde Sea, a fairly enclosed basin with a mean annual temperature of over 10° C. and minimum holothermic temperatures in the spring of 5–7° C. (Chumley, 1918, p. 14),[3] eggs and nauplii of *C. finmarchicus* were found by Nicholls (1933, p. 89) in January, followed by three well defined breeding periods, February–March, April–May, and June–July, with additional subsidiary broods of lesser importance in July and August. These broods represent successive generations, the adults dying off after spawning.

TABLE II

Relative percentages by number of *C. finmarchicus* to population of total region of Bay of Fundy, and of Gulf of Maine in 1932.

1932	Total region	Bay of Fundy	Gulf of Maine		
			Total gulf	Western area	Central area
April	25.7	24.6	26.8	34.9	18.7
May	56.4	52.4	60.4	66.0	54.8
June	44.7	47.5	41.9	44.4	39.4
August	35.6	29.8	41.4	39.4	43.3
September	37.3	30.7	43.9	43.2	90.2
April–September	39.9	37.0	42.9	45.6	49.3

In Norwegian waters off Möre, Ruud (1929) found propagation beginning in late March to early April, the maximum having passed by May both in 1926 and 1927. A second smaller maximum appeared in June and continued until late July. His records also indicate that spawning takes place earlier farther south and proceeds northward along the Norwegian coast where augmentation in spring and early summer (April–June) had previously been reported (Gran, 1902; Damas, 1905; Damas and Koefoed, 1907, etc.).

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[3] In the Gulf of Maine there is a mean annual temperature of 7–8° C. and a minimum holothermic temperature of about 2° around the margin.

In the region of Iceland, Paulsen (1906, pp. 19–20) reported the principal propagation season of *C. finmarchicus* to be from March until June with a possible second reproduction period later. At the meeting point of the Atlantic and North Polar currents, south of 67° N, the season is in late June (Damas, 1905), and east of Greenland, in late July (With, 1915).

In the Gulf of Maine and Bay of Fundy previous investigations have shown the stock of *Calanus* to be at a minimum in late February and early March, at which time regional differences would be expected to be least. Vernal augmentation begins in late March and April (depending on the locality) and varies somewhat from year to year. Early nauplii were abundant in Massachusetts Bay by April 7 in 1925 (Fish, 1928, p. 286). In coarse net hauls where the effect of production would not be noted until the new stock had reached copepodite stages, Bigelow (1926, p. 192) observed in 1920 a slight increase from March to April in the coastal zone between Cape Cod and Mt. Desert, a considerable increase in Massachusetts Bay (× 400), and in the western basin about the same average number as in February and March. His data of several years led him to conclude that probably the "multiplication of *Calanus* does not proceed so rapidly in the northern parts of the Gulf, though it may commence there as early as mid-April" (Ibid, p. 194). There was also evidence that breeding probably continues actively through June, and an increase in the numbers of *Calanus* in September suggested a second but less productive season in early autumn (Ibid, p. 207).

To the north Willey (1919) found evidence of vernal propagation over a broad area between Nova Scotia and Newfoundland. At the eastern end of St. Pierre Bank in 1915 *Calanus finmarchicus* in copepodite stages II–V was taken on June 24 and again on July 27. Stages III–VI were also obtained south of Cabot Strait on July 27 and in the Laurentian Channel on July 28.

Distribution of Eggs and Larvæ in 1932

An analysis of propagation in the Gulf of Maine and Bay of Fundy is complicated by the progressive delay in seasonal response from west to east (Fig. 3) combined with an active cyclonic drift, which renders it difficult to ascertain the source of larvæ taken in any particular locality. Fortunately an average difference of approximately one month in the time of spawning in the eastern and western parts of the Gulf made it possible to distinguish two breeding stocks, and, through the distribution of dominant developmental stages in successive months, trace in a general manner the rate of growth and subsequent dispersal in each.

April.—Spawning must have begun in March, and by late April had terminated over the greater part of the Gulf. The more advanced larvae had then reached the third and fourth copepodite stages, and were rather widely distributed in relatively small numbers. Nauplius stages IV–VI predominated in the Casco Bay region where production

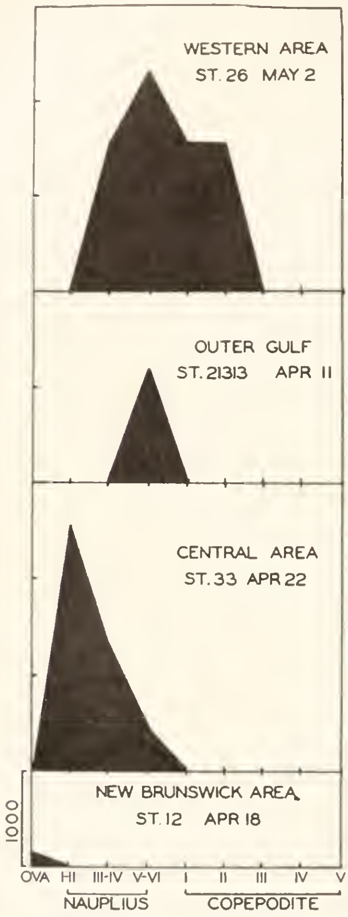


FIG. 3

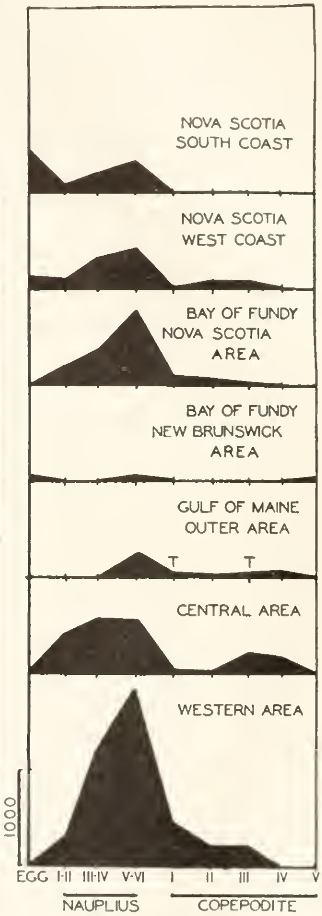
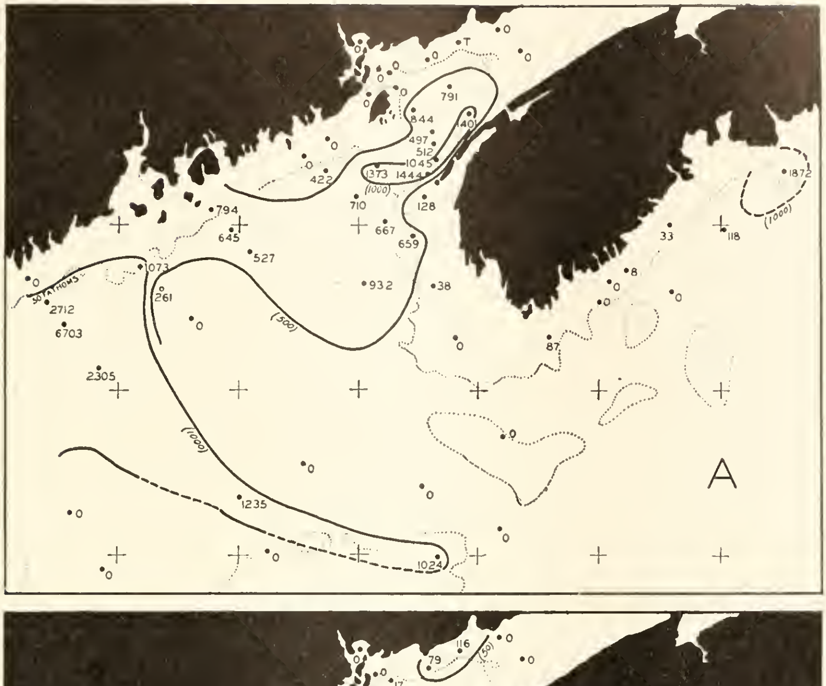


FIG. 4

FIG. 3. *Calanus finmarchicus*, showing progressive delay in seasonal response to the eastward, and evidence that the larval stock in the outer Gulf may have originated in the western area.

FIG. 4. *Calanus finmarchicus*, showing nauplius stages IV–VI dominating throughout the region in April 1932. (Average of all stations in each area.)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140
497
512
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527
667
659
1073
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932
38
1872
(1000)
33
118
8
50 FATHOMS
2712
6703
2305
87
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1235
1024
A
116
79
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151
3906
621
T
2187
350
218
50 FATHOMS
B

FIG. 5. Distribution of (*A*) nauplius stages V–VI of the western stock, and (*B*) eggs of the eastern stock in April 1932. Number per minute of towing.

was so much more extensive than elsewhere that its contributions outnumbered locally produced larvæ over the greater part of the Gulf and Bay, a condition which continued throughout the summer. As it was possible to make but one series of observations in the outer Gulf, the duration and extent of propagation in that area could not be determined, but the distribution of larvæ, consisting of nauplius stages V–VI with a small number of copepodite stage III and no intermediates, would suggest the western coastal area as the principal source. However, a local concentration of nauplius stages V–VI off Nova Scotia offers positive evidence that in outside waters the season was as far advanced off Halifax as in the region of Casco Bay. Between these two areas, along the south and west coasts of Nova Scotia, the season appeared to be less advanced, more closely corresponding to the eastern part of the Gulf where spawning had not yet ceased.

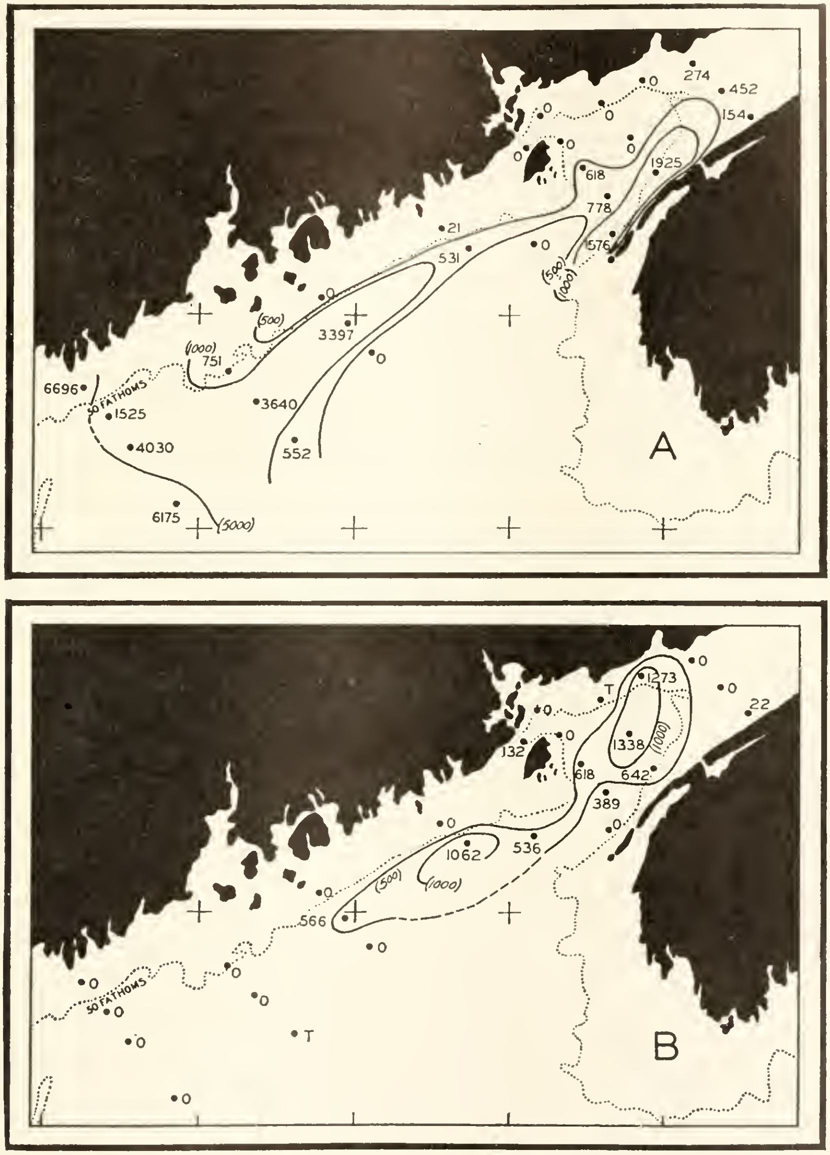
TABLE III

Maximal numbers of *Calanus finmarchicus* in various developmental stages in the western area of the Gulf and the Bay of Fundy, May, 1932.

Locality	1932	Ova	Nauplius			Copepodite				
			I–II	III–IV	V–VI	I	II	III	IV	V
Western area (Station 26)	May 31			1029	515	1029	3603	8234	4117	2058
Bay of Fundy (Station 36)	May 22	669	1338	6021	8697		1338	669		

Eggs of the eastern breeding stock were found in varying abundance in the central area, the Bay of Fundy, and off the west and south coasts of Nova Scotia, but not west of Mt. Desert or in the vicinity of Georges Banks.

May.—By the latter part of May, as indicated in Table III, the maximum numbers of larvæ from the western area were in copepodite stage III, and the local crop east of Mt. Desert, in nauplius stages V–VI, the latter corresponding to conditions in the western area a month earlier. In Fig. 6A late larval stages are seen to extend eastward from the rich Casco Bay region in a band whose inner margin parallels the 100-meter contour. They also penetrated in a tongue-like wedge into the Bay of Fundy along the Nova Scotian side, and show as a distinct second maximum in curves from both the Fundy and central areas (See Fig. 8, May sections off Cape Spencer, and Mt. Desert; also Table III, Station 36). These late copepodites of *Calanus finmarchicus* now dominated the total population and accounted largely for the tremendous rise in the volume of zoöplankton from April to May (Fig. 2).[4] Due to dispersal from the western area volumes everywhere increased, the increase being least noticeable in areas where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3397
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751
0
6696
50 FATHOMS
1525
3640
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552
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6175
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FIG. 6. Distribution of (*A*) copepodite stages IV–V of the western stock, and (*B*) nauplius stages I–II of the eastern stock in May 1932. Number per minute of towing.

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[4] Bogorov (1933) records an increase in the biomass (weight) of almost 60 times from copepodite stage I to stage VI.

the immigrant stock was not supplemented by a significant amount of local propagation. Particularly in the eastern coastal and New Brunswick regions, individuals of the local brood were not of sufficient size to be taken in the coarse meter nets in May.

Although late nauplii dominated in the eastern Gulf stock, some spawning must have continued well into May in the coastal waters east of Petit Manan, because early nauplii (I–II) were still numerous in this region between May 18 and 24 (Fig. 6*B*).

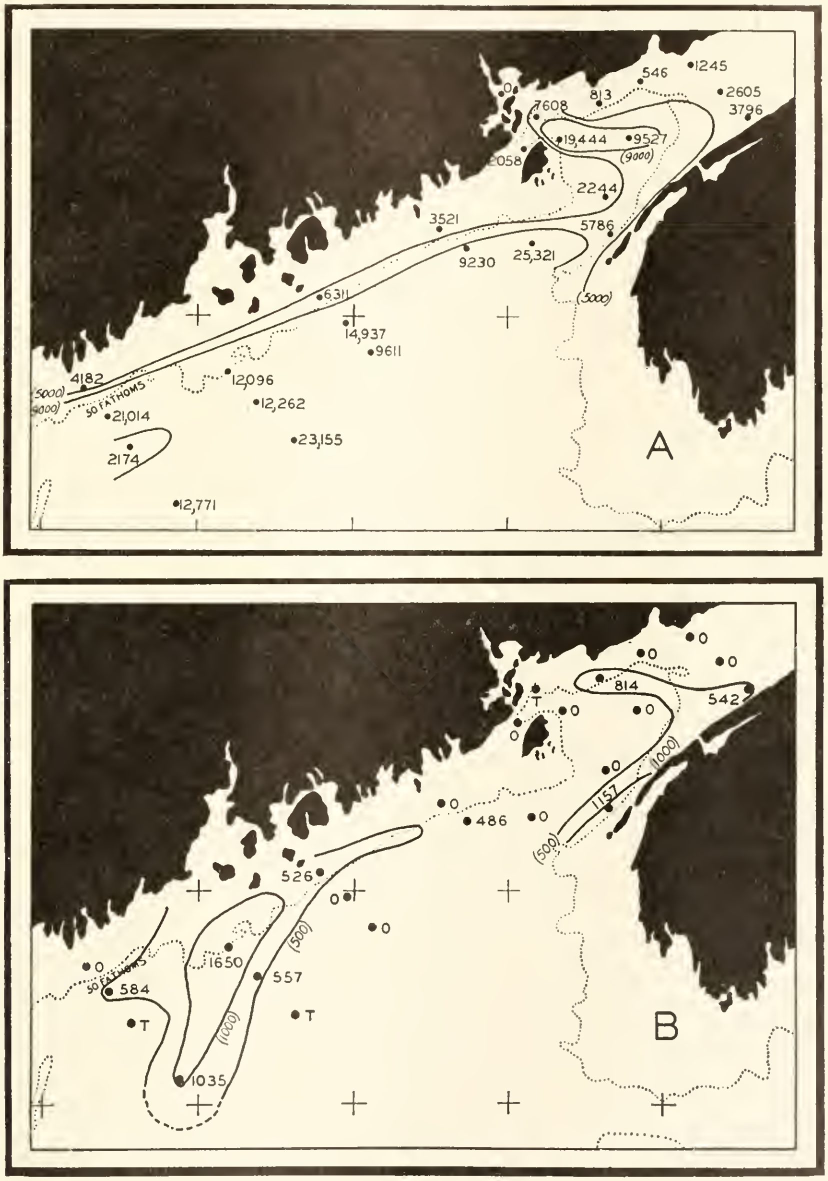
June.—The first western crop had matured and were spawning by June 20. As eggs were everywhere abundant (Figs. 7*A* and 8), although the eastern brood was still in copepodite stages (Fig. 9), the parent stock in all probability consisted almost entirely of individuals dispersed from the western area. In the eastern basin it is possible that the adults breeding at this time may have been reinforced to some extent from production centers outside of the Gulf, a possibility suggested by Bigelow's (1926, p. 209) records of a swarm of adults in this region on May 6, 1915, and the fact that in 1932 the largest single capture of eggs (25,321 per minute) was made in the eastern basin in June.

The eastern spring brood, in copepodite stages in June (Fig. 7*B*), were most numerous off Penobscot Bay and along the course of the drift into the Bay of Fundy. Elsewhere in the Bay and over the greater part of the central area, no copepodite stages were found, indicating that this brood suffers heavy mortality and only those produced in or transported as far as the western part of the central area survive.

August.—On August 11 in the Casco Bay region, after an interval of 42 days, the product of June spawning of the western stock, now in copepodite stages (III predominating), had been largely dissipated and the relatively few taken were distributed along the outermost stations from Casco Bay to the eastern basin. Elsewhere in the Gulf and Bay their numbers were extremely sparse.

Contrasted with copepodites of this second western crop, eggs and early larvae, assumed to represent spawning by the first eastern brood, were concentrated nearer the coast. Early nauplii were most abundant in the Bay of Fundy (Station 7) and central area, with smaller numbers occurring westward at the inner stations to Casco Bay.

September.—It was not possible to obtain data from the Gulf of Maine in September, but stations in the Bay of Fundy yielded small numbers of eggs, early nauplii (stage I) and copepodites in stage II. The latter were probably the product of August spawning by a second eastern brood and the former, in view of the absence of eggs and early larvæ along the New Brunswick coast and their concentration in the course of the drift along the Nova Scotian coast and over the deep basin in the Bay of Fundy on September 15, suggest a third generation of the western stock entering from the Gulf.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14,937
•9611
4182
(5000)
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50 FATHOMS
12,096
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(500)
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584
557
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1035

FIG. 7. Distribution of (*A*) eggs of the western stock, and (*B*) copepodite stages I–V of the eastern stock in June 1932. Number per minute of towing.

Comparison of Results in 1931 and 1932

In 1931 but one set of observations was made in the Gulf and Bay (August 21–September 5), corresponding stations being taken about two weeks later than in 1932 (August 8–21).

The season appears to have been about the same in the western area during the two years because a maximum of eggs between August 11 and 14 in 1932 would undoubtedly have hatched by August 21 when early nauplii were found predominating in 1931.

East of Mt. Desert, as the relative percentage of eggs and nauplii were much the same in the Nova Scotian and New Brunswick areas on August 17–21 in 1932 as on September 1–5, the spawning season would appear to have been about two weeks later in the previous year. Not having hydrographic data from the eastern basin of the Gulf for 1931, it is not possible to suggest a reason for the retarded season that year. Temperatures within the Bay of Fundy in 1931 and 1932 were not substantially different at this time.

Discussion and Conclusions

Throughout the Gulf of Maine and Bay of Fundy propagation begins in the spring and is followed by a succession of generations until autumn. The marked regional differences in the time when vernal augmentation begins are reflected through succeeding generations of that summer, irrespective of where the stock may become distributed. Thus by the time (June) that the spring crop from the western area has matured and spawned, it has become so generally distributed that eggs appear almost simultaneously throughout the Gulf and Bay (Fig. 8). Again, in July and August, when the smaller eastern vernal crop spawns, eggs appear in even greater abundance in the western area than in the region where the stock originates.

Propagation of the Stock Originating West of Mt. Desert in the Spring

The seasonal distribution of eggs and larvæ indicate two clearly defined spawning periods, March–April and June–July with a possible third in September. It is probable that most of the June–July brood remains in copepodite stages to form the bulk of the winter stock (maturing the following spring), but some appear to pass through the final moult and spawn in September.

The maximum numbers of larvæ of the first generation, which by late April in 1932 were in nauplius stages IV–VI, and in copepodite stage III thirty days later, had matured and were spawning by June 20.

The second generation is not as clearly traceable, but what are believed to have been members of the June brood were mainly in copepodite stage III forty-five days later on August 11 (Fig. 8, section *A*), and some became spawning adults by September 15 (p. 129).

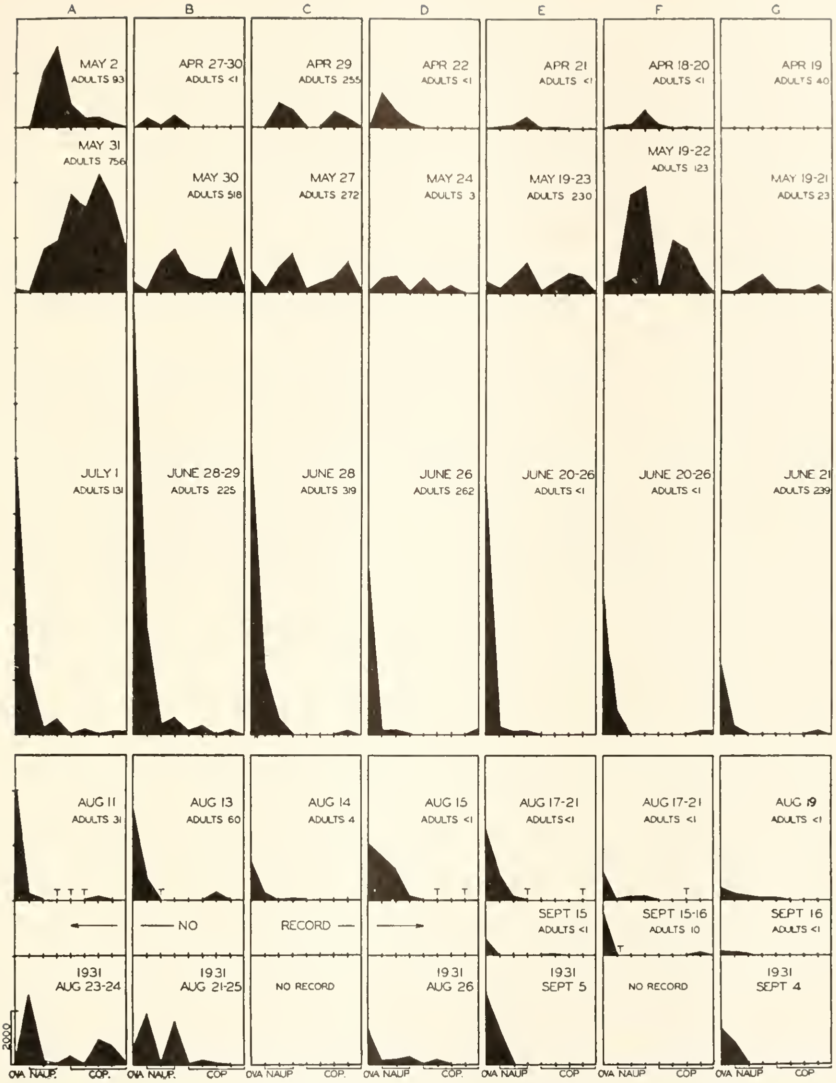

FIG. 8. Seasonal changes in the composition of the population of *Calanus finmarchicus* (developmental stages) in seven sections between Casco Bay and Cape Spencer.

Stages recorded as ova; nauplii I–II, III–IV, V–VI; copepodites I, II, III, IV, V.

Sections: *A*. Casco Bay (Stations 23*A*–25*A*, 26)
B. Penobscot Bay (Stations 27–29)
C. Mt. Desert (Stations 17, 30–31)
D. Moose Peak (Stations 32–33)
E. Passamaquoddy Bay (Stations 5–8*A*, 35)
F. Pt. Lipreau (Stations 13, 36–37)
G. Cape Spencer (Stations 9–11).
April–June: number per minute of towing.
August–September: number per cubic meter.

Propagation of the Stock Originating East of Mt. Desert

This region (Fig. 1) shows considerable local variation. In its western portion approaching Mt. Desert and on the Nova Scotian side of the Bay of Fundy (influenced by inflow from the outer Gulf), the spawning seasons of *Calanus* more closely approximated those to the west of Mt. Desert, while along the coast eastward from Petit Manan to St. John the seasons were progressively later. However, although evident, these local seasonal differences are relatively small, and for present biological purposes the waters east of Mt. Desert can be considered to comprise one region in which the maxima of corresponding stages of *Calanus* occur, as previously noted (p. 123), about four weeks later than in the western part of the Gulf.

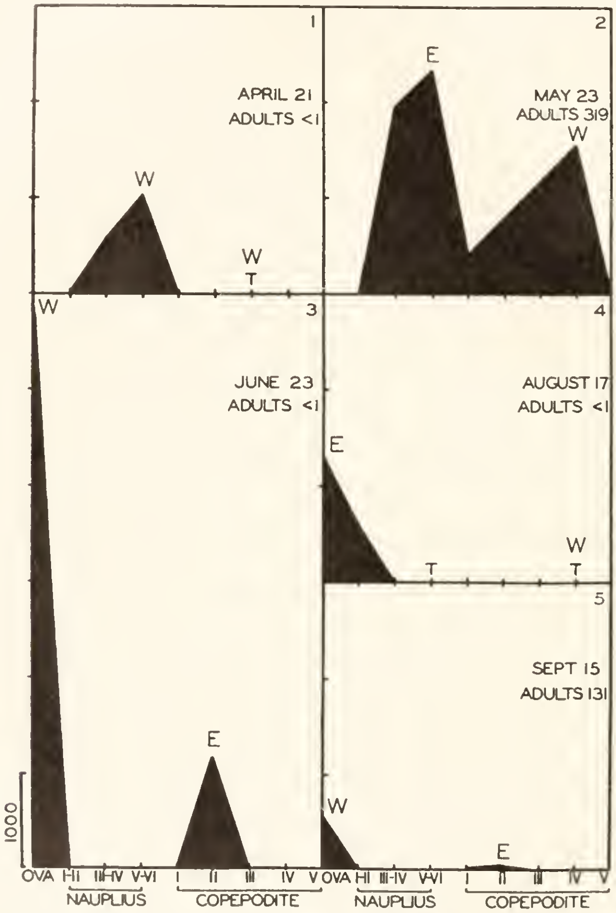

FIG. 9. Development of the western (*W*) and eastern (*E*) stocks as indicated at station 8.1 in the drift entering the Bay of Fundy in 1932.

There appear to be two less well defined propagation periods of the eastern stock, April–May and July–August. The first is indicated by eggs appearing along the New Brunswick coast on April 18 (Fig. 3) and the maximum of nauplius stages I–II in the vicinity of Petit Manan (Station 33) on April 22 (Fig. 3). Reaching nauplius V–VI a month later (May 18–24) and copepodite stages II–IV on June 20–26 (Figs. 8 and 9), it is presumed that August (8–21) eggs represent spawning by adults of this stock.

It was not possible to trace with certainty the outcome of July–August propagation because of the small number of individuals involved, but it would appear that the copepodites (stage II) entering the Bay of Fundy on September 15 (Fig. 9) were representatives of this crop. This second eastern brood of copepodites probably supplements the second and third (?) western broods to form the winter stock.

Rate of Early Development

In the Clyde Sea (Nicholls, 1933, p. 92) field and laboratory records have indicated a maximum of four weeks for the complete development of *Calanus finmarchicus*, a minimum of eleven days between the egg and first copepodite stage and intervals of three days for successive moults to stage V. A minimum of four days was required for the final moult to stage VI (adult).

Lebour (1916, p. 9), in laboratory experiments at Plymouth, found that nauplii appearing between April 17 and 24 reached copepodite stage V on May 19, having taken less than two months for development from the egg to the latter stage.

Off Möre Ruud (1929, p. 38) estimates that *Calanus finmarchicus* requires almost three months for its life cycle, allowing three to four weeks for embryonal development as indicated by Lebour (1916, p. 9). In 1926 there was about seven weeks between the maximum of nauplii and the maximum of stage VI.

In the Gulf of Maine the developmental period appears to be slightly less than in Norwegian waters, but, as observations at each station were taken at minimum intervals of one month, the rate of growth can only be approximated. In the case of the western stock, with an interval of 29–33 days between the maximum of copepodite stage III in May and the presence of eggs in June, followed by a period of 42–46 days before the next maximum of copepodite stage III, the complete cycle would appear to require from 71–79 days. Again, if eggs observed on September 15 (p. 129) can be considered to represent spawning by individuals incubating west of Mt. Desert from June 28 to July 1, and in copepodite stage III on August 11–14, then there must have been a maximum period of approximately 78–81 days between the second and third broods of eggs.

The two generations of the western stock would thus agree rather well and suggest a period of about two and one-half months from the egg to the spawning adult. Intervals between maxima of different stages also indicate about one month for the development from nauplius I to copepodite I, one-half month from copepodite I to copepodite V, and one month for the final moult, maturation, and incubation of the eggs. Although requiring confirmation by laboratory experimentation, there would seem to be about five days between moults in the nauplius stages and three days in the copepodite stages.[5]

No attempt has been made to calculate the developmental period of the eastern stock, hatching shortly before April 22 in the central area (Fig. 3, Station 33), because the greater part of this brood apparently matured and spawned in July, between cruises, and eggs taken in August no doubt represent late season spawning. This is indicated by the large percentage of nauplii up to stages III–IV in the central area on August 15.

August 15, 1932	Ova	Naup. I–II	Naup. III–IV	Naup. V–VI	Cop. I–V
Central area (Station 33)........	3,390	1,507	2,450	377	T

The rate of development would appear to be substantially the same as in the western stock, which is not surprising in view of the evidence that those members of the eastern spring brood reaching maturity probably entered the western area in early stages.

Rate of Late Development

As there was an interval of about one month between the maximum of late copepodite stages III–IV (May 27–31) and the next maximum of eggs (June 28–July 1) in the area west of Mt. Desert, it would appear that the time required for the ripening of the gonads and fertilization, after the moult to stage VI (adult), is probably about three weeks. A similar interval is suggested by the June brood if copepodite stages III–IV from the western region (August 11–13) formed the parent stock of early eggs entering the Bay of Fundy on September 15–16 (Fig. 8) in 1932. Nicholls (1933, p. 95) found that, having moulted to adults, there is a lag of from three weeks to one month before the next maximum of eggs in the Clyde Sea.

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[5] At Station 8.1 copepodite stage II predominated on May 15, and copepodite stage IV on May 23, nine days later.

Data on the interval between the final moult and the time of spawning in the winter stock are available only for the Bay of Fundy. Here the period must be considerably longer than in succeeding generations in the Gulf. The winter population, almost entirely in late copepodite stages in November, is indicated in Table IV and also in a haul by Willey in Passamaquoddy Bay (1921, pp. 187–8) on November 2 in 1916 when copepodites in stage V formed 46 per cent and adult females only 2 per cent of the total macrocalanid plankton. By February 23 in 1917 adult males and females formed 47 per cent and copepodite stage V, 36 per cent. In 1932, as shown below, the winter stock in this area had almost completed moulting (97.6 per cent adults) by March 21 and a considerable proportion of the females contained developing eggs. Yet no eggs were found in these waters on April 15, and spawning had apparently just begun on May 5.

Table IV

Winter population of *C. finmarchicus* in Bay of Fundy. Percentages by number of various developmental stages to total zoöplankton population.

Date	Copepodite		Adult	
1931–1932	Stage IV	Stage V	♀	♂
November 16	3.8%	96.2%	0%	0%
March 21	0%	2.4%	92.9	4.7

Relative Abundance of Adults

Accurate figures on the relative number of adults and individuals in developmental stages are difficult to obtain because the former cannot be sampled in a representative manner with the apparatus required for eggs and early larvæ. Copepodite stages IV and V, however, appear to have been sampled efficiently with both the coarse and fine nets, and by taking the fine net counts and applying the meter net data on the relative percentage of these stages to adults, it has been possible to obtain roughly comparable figures for the latter. Similar figures have been obtained for August and September using pump and meter net data but the error may prove to be greater in this case if it is found that late larvæ, particularly copepodite stage V, cannot be sampled representatively with the pump. There is some evidence that this might be true, in which event the mean number of both adults and late copepodite stages in the total region would be somewhat larger than indicated in Table V.

Mortality among Maturing Adults

In the Gulf of Maine during the interval between the maximum of late copepodite stages in late May and the maximum of eggs in June, there occurred the highest rate of mortality observed in zoöplankton during the year, the decline in the relative percentage of *Calanus* exceeding even the tremendous decline in the total volume of animal plankton (Fig. 2) at a time when the spring stock of this species, just reaching maturity, might have been expected to substantially increase the total plankton volume (p. 128).

A similar decline in *Calanus* in the Clyde Sea (where, as in the Gulf of Maine, the maximum of eggs coincides with the minimum of adult females) has been interpreted by Nicholls (1933, p. 95) to indicate that the period of maturation forms a very critical time during which the rate of mortality is so high that comparatively few females survive.

Support for this interpretation was found in the Bay of Fundy, where the eastern vernal crop was still in copepodite stages in late June. If the decline from May to June is not primarily due to a depletion of the food supply or unfavorable physical conditions, excessive mortality in the Bay would at this time be limited to immigrants from the west. Figure 2 shows this to be the case, as the eastern stock apparently offsets the decline of western immigrants in the Bay, and the mean volume continued to increase through June.

TABLE V

Number of *C. finmarchicus* in various stages of development, April to September, 1932.

1932	No. stations	Method	Eggs	Nauplii	Copepodites	Adults
April	27	Net	4	1615	406	128
May	26	Net	301	3013	4138	319
June	26	Net	8432	1850	307	311
August	27	Pump	2158	882	69	21
September	12	Pump	753	7	35	4

Net: number per minute. Pump: number per cubic meter.

Mortality in maturing adults of subsequent generations in the Gulf and Bay was not as clearly evident because both the meter net and pump data indicate that comparatively few survived to reach even copepodite stages. Perhaps the greater number of predatory plankton animals in summer served to keep the numbers down. Some such explanation is necessary to account for the large crop derived from the relatively small parent stock in March–April as compared with that in May–June.

Length of Life of Different Broods

The marked decline in the relative percentage of adults to copepodites following the breeding period in June (Fig. 10*A*), in view of the fact that in relation to eggs and nauplii, copepodites showed no significant increase (Fig. 10*B*), is considered to represent a dying off of adults after spawning. This appears to be a rather well-established characteristic of the species in the eastern Atlantic (Damas, 1905; Paulsen, 1906; Nicholls, 1933) and has been suggested by Bigelow (1926, p. 206) in the Gulf of Maine.

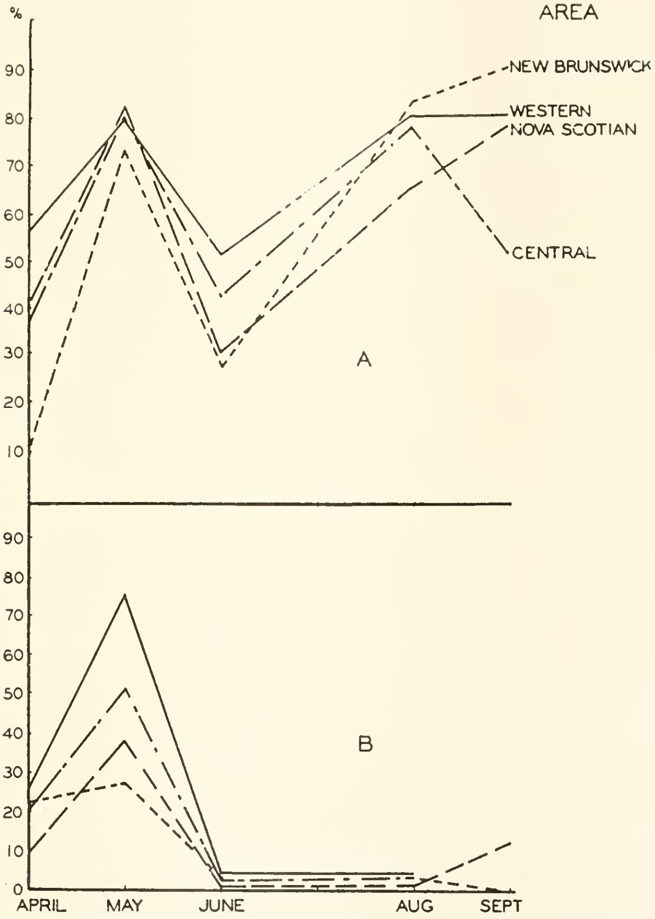

FIG. 10. *Calanus finmarchicus.* Seasonal changes in the relative percentage of (*A*) copepodites to adults, and (*B*) copepodites to eggs and nauplii.

The length of life of individuals produced in early April and maturing in June would thus probably not exceed four months, allowing two and one-half months for development to maturity and a maximum of one and one-half months of adult life (spawning adults in the western region on June 28–July 1 had greatly diminished by August 11–14 in 1932). A similar period of adult life in spring and summer (one and one-half months) has been found by Nicholls in the Clyde Sea area (1933, p. 92).

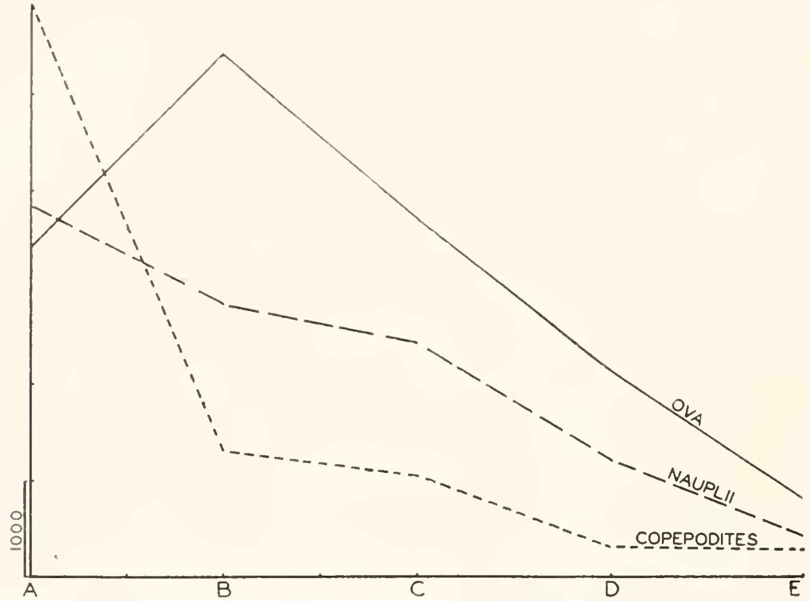

FIG. 11. Mean numbers of ova, nauplius and copepodite stages per minute of hauling for the period April–July, 1932.
Sections: *A.* Casco Bay, *B.* Penobscot Bay, *C.* Mt. Desert, *D.* Moose Peak, *E.* Cape Spencer.

In regard to the winter stock, in cold areas with fewer generations the length of life must be longer than in regions such as the Clyde Sea where spawning begins very early in the year. Our data would indicate that the increase in the number of copepodites in September, observed by Bigelow (1926, p. 207) in the Gulf and in 1932 in the drift entering the Bay of Fundy (increased from 0.3 per cent to 13.6 per cent, Fig. 10*B*), represented arrested development of at least a part of the June crop from the western region, supplemented perhaps to some extent by the July-August crop from the waters east of Mt. Desert. The small numbers of eggs of the third western brood in September do not appear adequate to account for the winter population. If the winter group then consists largely of individuals produced in June–August and dying off after spawning the following March–April, a maximum existence of approximately 10–11 months is indicated. September-spawned *Calanus* would have a maximum existence of 7–8 months. In the Bay of Fundy the period must be even longer (p. 133). Nicholls estimates (1933, pp. 91–92) the length of life of *Calanus* in the Clyde Sea area to be about nine weeks after the winter group has moulted to stage VI, with a total existence of 7–8 months.

There is some evidence that after reaching maturity the males of *Calanus finmarchicus* die off some time before the females. Referring again to Willey's records from the Quoddy region (1921, p. 187), of the adults taken on February 23 in 1917, males formed 20 per cent and females 27 per cent. In 1932 on March 21, a month later, females formed 92.9 per cent of the *Calanus* stock and males 4.7 per cent. Damas (1905) also found males of this species rare except at the epoch of vernal reproduction.

TABLE VI

Number of *C. finmarchicus* found in different stages in the New Brunswick area, April to September. April–June, number per minute. July to September, number per cubic meter.

New Brunswick area	Station	Ova	Nauplius			Copepodite				
1932			I–II	III–IV	V–VI	I	II	III	IV	V
April 15	5									
May 4	5	17								
May 19	5			31	62					
June 20	5	7608		507	507				T	
July 30	5	710	140							
August 21	5	375	187							
September 16	11A		40							

Relative Importance of Different Spawning Areas

The contention of previous observers (Willey, 1921, p. 189; Bigelow 1926, p. 207) that the western Gulf probably forms the principal source of supply of *Calanus* for the entire region, is borne out by the present investigations. At all times the coastal region west of Mt. Desert yielded the greatest numbers of eggs, larvæ, and adults, and the rapid decline to the eastward (Fig. 11), combined with the distribution of larvæ, designate it as the probable source of the major part of the population in the outer Gulf, the central area, and the Bay of Fundy. The fluctuations in the mean volume and relative percentage of *Calanus* in the total region in 1932 paralleled the seasonal changes in the western area (Fig. 2).

No doubt a portion of the individuals produced in the eastern region, particularly near its western margin, survive because the stock in very small numbers can be traced through successive generations. However, over the greater part of the Bay of Fundy and the turbulent coastal region of the Gulf east of Petit Manan, there is no evidence of any successful production of *Calanus*, although eggs transported west from the latter area may complete their development in a more favorable environment.

In the Quoddy region (Station 5), somewhat removed from the path of the drift from the Gulf, hauls throughout the season consisted principally of eggs with relatively few nauplii. Copepodite stages were taken during the summer with a meter net but their numbers were so small that almost none were found in pump and fine net samples. The large brood produced by immigrant adults in late June had apparently entirely vanished a month later, and eggs of the eastern stock in late July and August seem to have disappeared by September 16. A similar condition was found in August and September, 1931.

SUMMARY

1. *Calanus finmarchicus* is the most abundant pelagic animal in the Gulf of Maine, averaging 39.9 per cent of the total zoöplankton population during the period April to September, 1932, with a peak of 60.4 per cent in May. In the Bay of Fundy there was a mean of 35.5 per cent for the year 1931–32.

2. The adult stock varies markedly in abundance in different areas, the smallest numbers occurring in the Bay of Fundy and westward along the coast of Maine to Penobscot Bay. The population is everywhere at a minimum in late February and early March.

3. *Calanus* passes the winter largely in copepodite stage V, the final moult taking place in February and early March shortly before vernal augmentation.

4. An average difference of approximately one month in the time of spawning in the eastern and western parts of the region is reflected in succeeding generations after dispersal. Two breeding stocks can thus be distinguished.

5. In the western stock there are two well-defined breeding periods, March–April, and June–July, with a possible third in September, and two periods, April–May, and July–August in the eastern stock.

6. A developmental period of two and one-half months is indicated in both stocks, one month from nauplius I to copepodite I, one-half month from copepodite I to copepodite V, and one month for the final moult, maturation, and incubation of eggs.

7. Mortality is highest among maturing adults.

8. The total length of life in spring and summer does not exceed four months. The winter stock has a maximum existence of 10–11 months.

9. After reaching maturity the males die off before the females.

10. The western Gulf forms the principal source of supply of *Calanus* for the entire region. Propagation east of Mt. Desert in the inner Gulf is largely if not entirely, unsuccessful.

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THE INFLUENCE OF TEST MATERIALS ON REPRODUCTION IN PONTIGULASIA VAS (LEIDY) SCHOUTEDEN

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The test of *Pontigulasia vas* resembles that of *Difflugia* and other closely allied forms. It consists of small sand grains cemented together. These sand grains are collected by the animals before division and are formed into the new shell for one of the daughter individuals. According to Pateff (1926), Gruber in 1886 first described division in *Difflugia*. More recently work on division in these forms has been done by Zuelzer (1904), Goette (1916), Pateff (1926), and others.

While experimenting with *Pontigulasia vas*, it was found that reproduction could be prevented if the cultures were kept free of substances used in shell construction. Division here was apparently related to an inert foreign substance since the animals had plenty of food and were in good condition. It was then decided to investigate this problem further in the hope that the relationship between test materials, mitosis and division in this form could be made more clear.

The writer takes pleasure in expressing his appreciation for the interest of Dr. B. D. Reynolds, under whose direction this work was carried on.

MATERIAL AND METHODS

The *Pontigulasia* used in these experiments were collected from a number of ponds near Charlottesville, Virginia. After isolation and identification the animals were placed in Syracuse watch glasses with clean *Spirogyra*. These watch-glass cultures were kept in moist chambers. The *Pontigulasia* were examined daily, using the wide field binocular microscope, their number noted, and the water in the cultures changed. All water for these cultures was filtered and the pH value taken before using. During the time of the experiments the pH value ranged from 6.3 to 6.9. Where sand or other substances were used these materials were obtained as clean as possible and crushed in a mortar with water. The smaller fragments and water were then transferred to the cultures with pipettes. Observations on the *Pontigulasia* while gathering materials for shell building were made with the 16 or 8 mm. objective of a microscope. Both photomicrographs and camera lucida drawings were made of these animals. It was found that serial sections of *Pontigulasia* provided the only satisfactory method to show the protoplasmic structures within the shell. Sectioning was made possible by removing the silica dioxide (SiO_2) of the shell particles with hydrofluoric acid (H_2F_2). The *Pontigulasia* were fixed with Schaudinn's or Bouin's fluid and placed in a solution of 70 per cent alcohol and 2 per cent hydrofluoric acid. This solution was kept in paraffin-coated dishes. Ten to fourteen days were needed to remove the silica. Stronger solutions were tried but damaged fixation in most cases. Following removal of the silica, the *Pontigulasia* were imbedded in paraffin by the usual method.

TABLE I

The culturing of *Pontigulasia* without shell materials and the effect on division. Cultures with shell materials are the controls.

Culture number	Material for shell	Number of animals at start	Time	Number of animals at finish	Number of divisions
			days		
1	None	5	17	5	0
Control	Glass	5	17	10	5
2	None	5	21	5	0
Control	Sand	5	21	18	13
3	None	3	33	3	0
Control	Sand	3	33	30	27
4	None	5	26	6	1
Control	Sand	5	10	10*	5

* This was accidentally destroyed on the tenth day.

Sectioning was done at 7 μ. For staining Heidenhain's iron hematoxylin and Delafield's hematoxylin were used.

EXPERIMENTS

The following experiments were made to test the effect of culturing *Pontigulasia vas* without shell materials. The cultures were run in pairs, one was supplied with powdered sand or glass, the other was not. Beside this difference they were as far as possible exactly alike in composition and received the same treatment during the course of the experiments. The cultures with materials for shell building were considered the controls. Table I gives the results of these experiments in condensed form.

The only negative result was in Culture No. 4 where one division apparently took place. Two explanations can be given. This division was noticed the second day after the culture was started and the animal, which was small, might have been overlooked before. Secondly there might have been enough sand in the *Spirogyra* to furnish materials for the shell. This one negative result can hardly affect the conclusion that *Pontigulasia vas* will not divide unless materials for shell building are present.

It was then thought that perhaps mitotic division had taken place resulting in multinucleate forms similar to those produced in *Amœba proteus* by Chalkley and Daniel (1933). Sections of *Pontigulasia vas* which had been cultured without shell materials showed them to be in a normal condition. There was only one nucleus and, as far as could be determined, the cytoplasm appeared unaffected.

The rest of these *Pontigulasia* were given sand to determine if their power of reproduction had been affected. After some delay division took place. An individual from Culture 3 gave a typical reaction. This animal made no effort at first to collect shell materials but began to do so three days later. By the fourth day it had produced a normal offspring. It appears, therefore, that the power of reproduction had not been permanently affected.

During the experiments the actions of the *Pontigulasia* without shell materials were interesting. Much of the time was spent moving about on the bottom of the watch glasses without any attempt to feed. At such times the pseudopods would become ragged in outline with a wide hyaline area at the ends. This type of pseudopod is usually associated with the collection of test materials. Undoubtedly these animals would have collected sand had it been present. After a day or two of such moving about the animals would begin to feed again. At other times they would go into a quiescent state for several days before feeding.

Some work was done in an attempt to find when nuclear changes began in relation to the sand-gathering activities. A number of *Pontigulasia* were isolated for several days with plenty of food. Finely ground sand was then added and the animals began to collect it. The gathering of shell materials took from 4½ to 5 hours. The pseudopods were next withdrawn and the new shell was formed immediately in front of the mother individual. During this process a number of animals were fixed and sectioned. It was found that mitosis was not begun until the collecting process had been almost completed. Fig. 1, *a*, shows a section of a *Pontigulasia* which had been collecting shell materials for four hours at the time of fixation. Nuclear changes here are very slight. There is, however, a decided mass of chromatin in the center of the nucleus. The sand grains in this form are collected and held outside the mother shell similar to *Difflugia urceolata* as found by

Zuelzer (1904). The vacuoles in the cytoplasm protruding from the shell are pockets from which the sand grains have been dissolved.

Fig. 1, *b*, shows an animal which was fixed just after the collection of shell materials had been completed but before the new shell was formed. The nucleus in this animal is in middle prophase, showing the chromatin granules on a definite linin reticulum. At this stage the nucleus is much enlarged. This section also shows the details of the shell very clearly. The internal diaphragm can be seen with the cytoplasm flowing through it. This diaphragm is easily overlooked in the living condition and the constriction between the neck and the fundus is not as deep in these forms as described by Penard (1902), Cash (1909), and Kudo (1931).

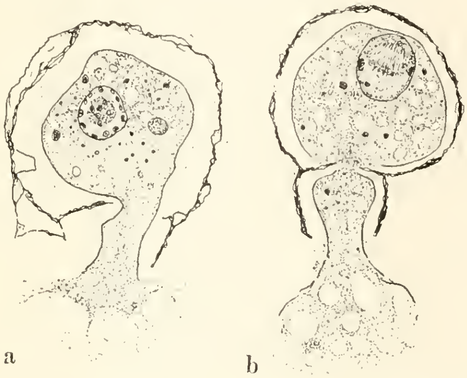

FIG. 1. Sections of *Pontigulasia vas* fixed while gathering test materials before division. Camera lucida drawings. × 400.

a. This animal had been collecting fine sand grains for 4 hours. The shell in this section was distorted when sectioned. Note that the nucleus is scarcely changed from the resting condition.

b. Section of an animal which had been collecting for 4½ hours. The formation of pseudopods had ceased but the sand grains were still a formless mass in front of the mouth of the old shell. The nucleus is much enlarged and shows a definite linin reticulum with the chromatin granules. Middle prophase. Note the constriction and the internal diaphragm in the shell.

The process of division was not investigated further in this study, but it seems fairly certain that nuclear division is not started until the collecting of shell materials has been completed. A more complete study of binary fission in this form is being made.

DISCUSSION

As far as these experiments on the control of division in *Pontigulasia vas* were carried, it seems that reproduction can be prevented by withholding shell materials. It is possible that division might have taken place if the cultures had been continued over a longer period of time. This appears unlikely, however, for in some uncontrolled cultures animals have been kept as long as 70 days without reproduction.

There still remains the possibility that individuals without shells were produced. If so, these naked animals could not have survived long for no evidences of them were seen.

No definite effect of inhibited division can be given here. There was a tendency for animals in cultures without sand to go into an inactive state immediately after attempting to collect shell materials. This inactive state lasted from one to three days. Also when such individuals were given sand there appeared to be a delay in collecting it and a delay in division afterwards. The animals resulting from such division, however, were normal as far as determined. If these variations are a result of inhibited division then they must be considered in the work done on nuclear division and its relation to shell materials. The animals used in this part of the work were kept without sand for a short time only in an effort to reduce any error from this source.

Nuclear division in *Pontigulasia vas* appears to be dependent on the shell materials in the cytoplasm rather than the collecting action of the animal itself. As the collecting process nears completion, the *Pontigulasia* seem to be in a very unstable physiological condition. If they are disturbed in any way the sand grains will be discarded and division will not take place. Pateff (1926) found the same condition in *Difflugia mammallaris* when about to reproduce.

Summary

1. *Pontigulasia vas* will not reproduce unless shell materials are present in the cultures.

2. The shell materials are collected for a period of approximately four hours immediately before division.

3. There are no changes in the nucleus until almost all the shell materials are collected. Nuclear changes are begun, however, before the formation of the new shell.

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THE WASTING DISEASE OF ZOSTERA MARINA[1]

I. A PHYTOLOGICAL INVESTIGATION OF THE DISEASED PLANT

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At the time that this study was begun, mid-summer, 1933, an epidemic, wasting disease had almost entirely destroyed the normally dense beds of the common marine eel-grass, *Zostera marina*, along the Atlantic coasts of North America and Europe. Since the plant is a prominent member of the shallow water community, sheltering a variety of larval and small sea animals, serving substantially as food for migratory game birds, and checking erosion of the bottom, and because the leaves are used for packing, upholstering, insulating, and other commercial purposes for which they are often peculiarly desirable, the disappearance of the eel-grass became a problem of practical concern. It was recognized, too, that the extensive and persistent wasting of *Zostera* might indicate phytopathological relationships in the sea as significant as the more evident conditions in land vegetation. A further impetus was given this investigation by the popularization of a number of physical and chemical hypotheses for the depletion, of restricted application, and usually of a prejudicial nature.

In France, Holland, Denmark, and Canada investigations were already in progress (6), (7), (11), (13), (18). The history of the disease here and abroad, the current condition of the eel-grass beds, and the economic and ecological implications of the wasting have been developed by a number of observers, and comprehensive summaries have been published from time to time by the U. S. Bureau of Biological Survey and others (1), (3), (6), (7), (10), (14).

This paper is an account of investigations upon the pathology of the diseased eel-grass and upon the specific parasitic nature of an unidentified *Labyrinthula*, an amœba-like organism with mycetozoan affinities, found in the leaves of the plant over the entire range of the epidemic condition. They were begun in the attempt to extend the findings of Messrs. Fischer-Piette, Heim, and Lami of France, who early reported a pathogenic bacterium as active in the diseased *Zostera* beds of their coast, and the work of Dr. H. Petersen of Copenhagen and Miss I. Mounce of Ottawa, who had studied a fungus associated with the wasting in Danish and Canadian waters, respectively (6), (11), (12). To these ends our results were negative (16). None of the bacteria isolated from diseased plants demonstrated pathogenic behavior in inoculation tests, and we failed to find any satisfactory physical association with the symptoms in leaves, stems, or roots in a variety of live specimens taken from the neighborhood of Woods Hole, Massachusetts, or in preserved material sent from many points along the American coast. From further exchange of specimens we were able to establish that though the fungus, *Ophiobolus halimus*, a new species described by Mounce and Diehl, was undoubtedly very abundant along the Canadian, Danish, and English coasts, it appeared only infrequently in diseased eel-grass taken from representative beds running from the coast of Maine southward, and that its development in these sporadic cases was incomplete (12), (17).

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[1] Submitted as a thesis in partial fulfillment of requirements for degree of Doctor of Philosophy, Rutgers University, 1935.

[2] Contribution No. 90, Woods Hole Oceanographic Institution, and Journal Series Paper of the N. J. Agr. Exp. Station.

Nevertheless, the symptoms of the wasting disease were identical in the exchange specimens and preparations taken from many parts of the American and European coasts, and these have remained constant through the epidemic's duration.

Investigation of the Diseased Plant

The foliage of infected beds is represented by short, young, rapidly growing, green leaves, eight to twelve centimeters in length, which may not show the splotching and dark streaking characteristic of the disease; by leaves of medium development, badly discolored and spotted with brown or black; and by the wilted basal fragments of older leaves sloughed away shortly after the disintegration of the greater part of their green tissues. Usually, the spotting is confined to the leaf epidermis, but it may arise in any green tissue, develop into plaques of considerable area, or form mosaics of widely scattered, darkened cells. The streaking, due to the serial infection of the larger, longitudinally disposed mesophyll cells, is more striking; it is generally sharply bounded by the longitudinal mesophyll spaces and terminated by the lateral septa (Fig. 1). Its margins spread rapidly, from three to five centimeters a day, or at higher rates during the warm summer when the disease is most active.

Often, but not always, the cuticle and cortex of the stem are irregularly pigmented with dark brown and black; the condition is not uniformly associated with the disease. After defoliation of the plant, through local activity of the parasite in the leaves, the stem may persist for one or two seasons to produce several new shoots each season —manifest in the apparent redevelopment of wasted beds. Eventually the reserve food supply, the accumulation of which has been limited by wasting, is exhausted and the weakened stems are invaded by bacteria, fungi, and attending saprophytes; the less vascular true roots decompose rapidly in the mud.

Microscopic examination of discolored and streaked tissue does not always reveal the protozoan parasite with which this paper is concerned; the reason for this discrepancy will be clear when its life history is described. On the other hand, the changes that can be directly observed following experimental infection of the leaf are identical with those given by histological preparations of the diseased plant from naturally infected beds. Within a few hours after the cells have been entered by the parasite, the chloroplasts become olive or straw colored and either pass to the periphery of the cell where they adhere in masses, or coalesce near the center. Soon afterward they fragment, and the small particles of chloroplast material become dispersed through the cell or coagulate into dark, irregular clots. The nucleus persists for a long time after the breakdown of the chloroplasts, but finally takes on a spherical outline, granular texture, and brown or black pigmentation; often it is hidden by debris. In addition, dark globules of resinous substances, apparently produced by the injured cell itself, and not identified with any of the broken structures, make their appearances at intervals along the cell wall.

No changes attributable to the direct attack of the parasite upon the stems and roots could be observed; though the organism was found in the cells of the stem cortex several times, it was never abundant there nor uniformly associated with the discoloration of those parts. The damage brought about by the protozoan parasite is reflected systemically only through the destruction of the plant's food-synthesizing mechanism. Death follows the loss of the photo-synthetic function of the green leaves and the exhaustion of reserve food in the stems.

Demonstration of the *Labyrinthula* in Diseased Eel-Grass

The spindle-shaped cells and interconnecting system of pseudopodia peculiar to the *Labyrinthulæ* and characteristic of the protozoan parasite of eel-grass were first observed in stained sections of diseased leaves taken from the region of Nantucket, Massachusetts, in the spring of 1934. That they had been overlooked until this time may have been due to their unusual distribution in the infected leaf, always well in advance of the visible streaking and discoloration itself, in the green tissues beyond the borders of the visible symptoms, and also to their very slight refractility. Their staining, too, is highly specific and difficult; to date a sequence of safranin, carbol-thionin, and Orange G. dyes, though uncertain in results, has given the best demonstration in formol-acetic-alcohol preserved field specimens. In these stained preparations the spindle-shaped cells of the *Labyrinthula* occurred with such persistence and were so suggestively oriented in the pathological picture that they were singled out as being in some way related to the disease.

A careful re-examination of specimens received from many points south of Woods Hole to Beaufort, N. C., confirmed the association in those waters, and later, when specimens were exchanged with Miss Mounce in St. Andrews, N. B., and with Mr. Tutin in Plymouth, England, the relations in these regions became evident also. Not long afterward, viable *Labyrinthulæ* were demonstrated by these investigators in their respective localities. Working independently, Mr. Loraine Young observed and studied the organism in diseased leaves in returning beds near Mt. Desert Island, Maine.

Dr. Frederick S. Sparrow of Dartmouth College identified the organism as one of the *Labyrinthulæ*, a family of the *Proteomyxa*, characterized by peculiar interconnecting pseudopodia; he also made many valuable suggestions relating to the culture and manipulation of the form.

In many features the *Labyrinthula* in *Zostera* is similar to the members of the family that have been described as parasitic upon marine algæ (2), (4), (9). It is not possible to identify the species with any of these at this time, however.

As found in the infected plant, the *Labyrinthula* may appear as a number of individual, colorless, slightly refractile, spindle-shaped cells from 10 to 20 mμ long and 5 to 8 mμ in diameter, distributed at random or in unrelated clusters through the green tissues and longitudinal spaces of the mesophyll (Fig. 2); as net-like aggregates of individuals connected together by a web of fine, branching, terminally secreted pseudopodia similarly disposed in the parasitized leaf (Fig. 3); as pseudoplasmodia formed of many individual spindles closely massed into thick cords or less regular strands (Fig. 4); or as what is apparently a resting stage—cyst-like, spherical bodies, about 6 to 10 mμ in diameter, light brown in color, surrounded by less dense, thick cuticles, and occurring mixed with residues in the older infected tissues (Fig. 5).

The spindles are motile; in the net-like organization they glide along the interconnecting pseudopodial web at rates in excess of 50 mμ per minute. They tend to concentrate in newly invaded tissue entrance to which is gained by local digestion of the cell walls. The pseudoplasmodium, sometimes relatively massive, also performs progressive movements, though not so rapidly as the individual cells; terminal masses of spindles may disperse to form a number of more lively branches (Fig. 6).

FIG. 1 FIG. 2

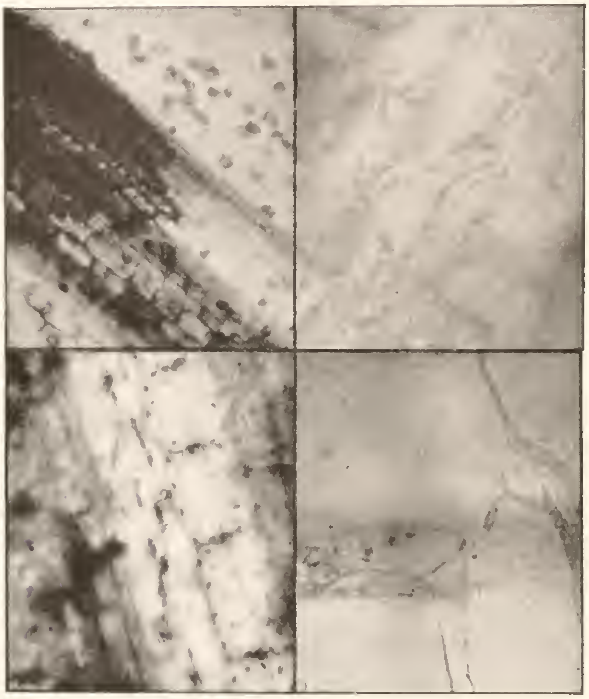


FIG. 3 FIG. 4

PLATE I

FIG. 1. Typical symptoms of the wasting disease in the leaf. Note extensive streaking in the mesophyll and mosaic infection of epidermal cells at upper right. Photomicrograph of clove oil-cleared preparation, stained with safranin—× 410.

FIG. 2. Individual spindle cells of *Labyrinthula*. No evidences here of pseudopodial net-structure. Photomicrograph from living preparation—× 480.

FIG. 3. Chain-like aggregates of connected spindles extending through longitudinal mesophyll space at boundary of infected zone. Note broken tissue at left and healthy mesophyll cells at right. Photomicrograph of living preparation—× 445.

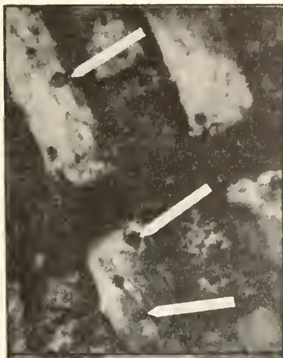
FIG. 4. Pseudoplasmodium in recently infected mesophyll tissue. Note early stages of degeneration in invaded mesophyll cells at left. Photomicrograph of living preparation—× 480.

All of these behaviors may be observed readily in damp cell mounts of excised, living, infected leaf tissue, and they may be extended to the study of the parasitic behavior by adding fresh, uninfected tissue to these mounts, a procedure giving an excellent demonstration of the migration of spindles and pseudoplasmodia into new host tissue.

Demonstration of Parasitism

A method of this design was employed in establishing the parasitic nature of the *Labyrinthula* found in eel-grass. Thirty-six damp cell,

Fig. 5



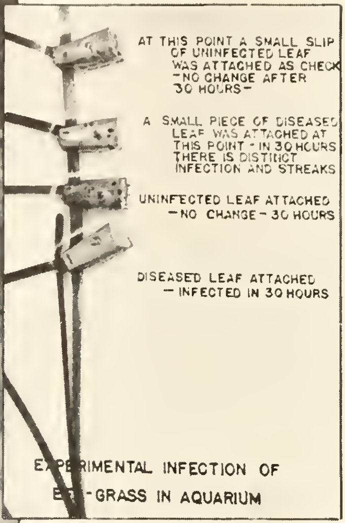

AT THIS POINT A SMALL SLIP OF UNINFECTED LEAF WAS ATTACHED AS CHECK —NO CHANGE AFTER 30 HOURS—
A SMALL PIECE OF DISEASED LEAF WAS ATTACHED AT THIS POINT - IN 30 HOURS THERE IS DISTINCT INFECTION AND STREAKS
UNINFECTED LEAF ATTACHED —NO CHANGE – 30 HOURS
DISEASED LEAF ATTACHED — INFECTED IN 30 HOURS
EXPERIMENTAL INFECTION OF EEL-GRASS IN AQUARIUM

Fig. 7

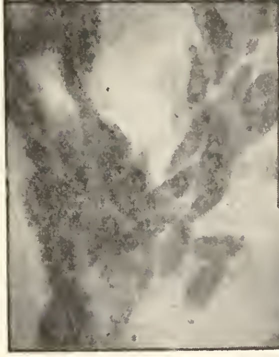


Fig. 6

Plate II

Fig. 5. Cyst-like resting stages of the *Labyrinthula* lying in mesophyll spaces of blackened leaf—× 475.

Fig. 6. Dissolution of pseudoplasmodium into individual spindles. Here the centrally placed "vacuoles" may be distinguished. Photomicrograph of living preparation—× 1520.

Fig. 7. Demonstration of inoculation method. This leaf was taken from an aquarium experiment. In the field the inocula and checks were spaced at intervals of about four inches. Here the tendency of inocula to overrun checks is already evident.

hanging drop preparations were made, each consisting of small tangential sections of living, uninfected leaf held against the under side of the cover-glass by a thin droplet of Seitz-filtered sea water in which, at a distance of about a millimeter from the test tissue, small pieces of diseased leaf containing viable *Labyrinthula* cells were also placed. Care was taken to avoid serious contamination.

Within eight to forty-eight hours the migration of motile spindles or the extension of pseudoplasmodia from diseased sections to the originally uninfested tissue and the invasion of the new host was evident in these preparations.

In these recently invaded pieces of host tissue the progress of disintegration was identical to that observed in sections of the living, diseased leaf studied earlier. Thirty of these preparations gave positive results, while ten check mounts containing green tissue and no "inocula" demonstrated that the tissue might be maintained in good health under the conditions of the experiment. One of these checks became contaminated by an air fungus, but nowhere else in these tests did mycelium appear.

Field Inoculation Experiments

That damp cell inoculation of excised leaf tissue may not indicate the parasitic habit of the *Labyrinthula* in nature is quite clear, but this criticism is effectively answered in field experiments repeated a number of times in selected eel-grass beds in Hadley's Harbor, near Woods Hole, during the summers 1934 and 1935.

Here healthy plants were inoculated by fastening to their leaves small pieces of diseased leaf, and check preparations were made by attaching pieces of normal leaf, or bits of paper to the same leaves. These were usually arranged alternately as shown in Fig. 7. Small cork clamps held the inocula and checks in place and great care was exercised to avoid mechanical injury of the test plant. The experiments during late summer, 1934, were directed toward determining the validity of this technique and adapting it to the more detailed studies that followed, but it was notable in these preliminary trials, repeated four times in two separate beds, and using from twenty to twenty-five tests in each trial, that the plants uniformly showed a degree of infection greater than 50 per cent at the end of two days exposure. The inoculated leaves were always marked with the symptoms of the disease generating from the points at which the inocula were attached, and the checks were free from any discoloration. Sections prepared from the newly infected leaves invariably showed heavy invasions of viable *Labyrinthulæ*, and of no other form, and such sections attached to clean plants brought about infection in the new host.

During the following summer, 1935, the field inoculation experiments were resumed in more detail to confirm the impressions of the previous year and to discover why the *Ophiobolus* did not appear in the inoculated leaves, though the mycelium had been found occasionally in specimens taken from nearby beds. The same method of inoculation was followed; about sixty inoculations and checks were prepared each time, but instead of removing all of the plants at the end of two days as before, part were removed and examined after one day, another aliquot after two days, and the remainder after three days.

These specimens were studied very closely; free-hand sections were prepared from both checks and inoculations and inspected under the high dry powers of the microscope. Table I summarizes the results of this search.

Table I

Incidence of Labyrinthula and other Invaders in Field Inoculation Experiments on Zostera marina

	Specimens taken after		
	24 hrs.	2 days	3 days
Number of inoculations	14	13	25
Number of inoculations showing symptoms of disease	8	9	22
Number of inoculations showing invasion by *Labyrinthula*	7	9	22
Number of inoculations showing invasion by bacteria or fungi	0	0	3‡
Number of checks	14	9	22
Number of checks showing streaks or other symptoms	0	3*	12†
Number of checks showing invasion by *Labyrinthula*	0	2*	11†
Number of checks showing invasion by bacteria or fungi	0	0	0

* Two of these checks were obviously overrun by extensive streaking arising from the inoculated region of the same leaf. The third showed some slight disintegration but no invaders—perhaps mechanical injury.

† In ten cases there was evident overrunning of the checks by extension of streaking from the point of inoculation. One is a case of spontaneous infection, clearly, because the inoculation in that case did not "take."

‡ The form appeared to be a filamentous fungus, but the mycelium was very poorly developed and mixed in with *Labyrinthula* and debris.

Later in the summer the experiments were repeated with some modifications that were designed to cut down the tendency of inoculations to overrun the check areas. These changes were not successful, but the results obtained confirm those secured earlier in the season and are given in Table II.

TABLE II

Incidence of Labyrinthula and other Invaders in Field Inoculation Experiments on Zostera marina

	Specimens taken after		
	24 hrs.	2 days	3 days
Number of inoculations	14	13	24
Number of inoculations showing symptoms of disease when removed	9	9	21
Number of inoculations showing invasion by *Labyrinthula*	10§	11§	22
Number of inoculations showing invasion by bacteria or fungi	0	0	3‡
Number of checks	14	10	22
Number of checks showing streaks or other symptoms	0	3*	12†
Number of checks showing invasion by *Labyrinthula*	0	3*	12†
Number of checks showing invasion by bacteria or fungi	0	0	0

* Despite wider separation of checks and inoculations the three checks were in these cases completely overrun by the development of streaking from the point of inoculation.

† In nine cases there was obvious overrunning of the checks. In two cases the check had become infected and had inoculated the leaf to which it was attached; in the other, the leaf had become infected and had inoculated the check.

‡ Fine mycelium in two cases; in the other distinctly heavy development of thick-walled mycelium.

§ Indicating infection so recent that the recognizable symptoms of darkening have not yet developed.

CULTURE OF THE *Labyrinthula*

A number of unsuccessful attempts have been made to transfer the *Labyrinthula* from its host to artificial media of various types. We have also tried species of *Bryopsis* and *Cladophora* as substitute hosts, choices determined by previously reported parasitism of these algæ by *Labyrinthulæ* (4).

Large numbers of spindles and masses of pseudoplasmodia free of obscuring host tissue could be obtained for morphological study, however, by culturing small pieces of infected leaf in sealed, hanging drop mounts. When these were exposed to diffused daylight for a few hours at room temperatures, the spindles migrated to the periphery of the supporting droplet in cord-like aggregates, sprays, and clusters.

If desired, the cover-slip could be lifted from the slide and the host tissue removed with fine tweezers to leave an unobstructed field in which the movement and development of the *Labyrinthula* might be studied over a period of four to eight hours.

Some cursory inoculation experiments using the isolated *Labyrinthulæ* as inocula have not given satisfactory results.

Discussion and Summary

From distribution studies on specimens representing the range of the wasting disease and taken over a period of three years, it appears that the *Labyrinthula* demonstrated in these experiments is universally associated with the epidemic condition. It did not occur, for example in eel-grass from Nanaimo, B. C., where the wasting disease has not been active, though the leaves of the plants were marked by lesions of various bacteria, fungi, and worms.

Histological examination and inoculation experiments indicate the true parasitic nature of the *Labyrinthula.* The rapidity with which it produces infection in these experiments is commensural with the aggressive habit of the disease in nature, and in this respect it seems to be the only organism transferred in a time interval comparable with the severity of the disease. The appearance of mycelium after several days, following the invasion by *Labyrinthulæ*, suggests a minor rôle for the fungus. Until this is definitely established by inoculation tests in the more northern waters the alternative of two active parasites must be considered. This does not seem likely, however, for the *Labyrinthula* is present and viable in the middle Atlantic regions throughout the year—it is notably more destructive in the late summer. If the *Ophiobolus* be a true parasite, its parasitic habit must be restricted to the Canadian and north European waters where it reaches its complete development.

From its observed behavior the *Labyrinthula* appears to be a specific parasite of *Zostera marina* L., but until the life history of the organism has been studied more carefully, it seems unwise to constitute a new species. It will be necessary, too, to attempt further host transfer experiments and to search for the *Labyrinthula* among related plants and members of the same community.

No attempt has been made in this paper to consider pertinent epidemiological problems; these are reserved for a report on field studies now in progress.

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CRUSTACEAN EYE-STALK HORMONE AND RETINAL PIGMENT MIGRATION

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INTRODUCTION

The study of color changes in crustaceans has for a long time occupied the efforts of investigators. Pouchet (1876) believed such changes to be under the control of the nervous system. But Perkins' (1928) and Koller's (1928) independent discoveries that a hormone, which effected concentration of the body pigment, was elaborated in the eye-stalks of *Palæmonetes* and of *Crangon* opened a fertile field for research. Since that time a series of studies by Koller and Meyer (1930), Smith (1930), Kropp and Perkins (1933), Hosoi (1934) and Hanström (1935) has shown this hormone to be present in a large number of crustaceans.

Brown (1935) has offered the opinion that more than one chromatophore-activating hormone is secreted from the eye-stalk into the blood stream. Hosoi's report (1934) that extracts of various organs of *Penæus japonicus* were able to concentrate the chromatophores of blinded *Paratya compressa*, might be taken to indicate that the chromatophore reaction is merely a non-specific reaction to protein extract. But I am inclined to attribute his results to traces of the hormone present in the blood coagulated within the various organs used.

This heretofore entirely physiological problem was given a morphological aspect by the discovery in crustacean eye-stalks of certain structures which seem to be secretory in character (Koller, 1930; Hanström, 1931, 1935; Sjögren, 1934). Brown (1933) and Hosoi (1934) reported the presence of the chromatophore activator in extracts of the ventral nerve cord, but no histological study of this organ has been made to discover similar secretory structures.

The complexity and similarity of this eye-stalk hormone to the pituitary secretions of vertebrates had already suggested itself to Kropp

(1932), who investigated the possibility of a gonadotropic effect on immature rats. In 1934 Navez and Kropp found that the crustacean chromatophore activator possessed growth-promoting properties when tested by means of decapitated coleoptiles of *Avena*.

Closely related to this subject of control of crustacean body pigments has been that of retinal pigment migration. The literature, up to within recent years, deals almost exclusively with such topics as the effects of light intensities, spectral colors, and temperature on the rate of migration. Only recently (Bennitt and Merrick, 1932) has there been any attempt to approach this problem from a chemical basis.

Parker (1897), in his study of the structure and positional changes of the retinal pigment cells in *Palæmonetes,* could find no nerves supplying the distal pigment cells. Still, the problem of interrelationship of the eyes, a controversial and as yet unsettled one in vertebrate physiology, was brought into the discussion. The crux of the problem, briefly, is this: that if retinal pigment migration is independent of the central nervous system, then the condition of the pigment in the light-adapted eye of an animal should have no effect on that of the other covered eye of the same animal, and, conversely, if the central nervous system is involved in retinal pigment migration, one eye may be "sympathetically" influenced by the condition of the other eye. The evidence presented on this point has been inconclusive. Parker (1897) believed from his experiments with *Palæmonetes* that retinal pigment migration was independent of the central nervous system, but von Frisch (1908) using *Palæmon* could get no decisive results. Castle (1927), who confined his study to the proximal pigment cells in the eyes of *Palæmonetes,* concluded that an illuminated eye was without "sympathetic" effect on the position of the pigment in the covered eye. Bennitt (1924, 1932*a*) worked with several decapod crustaceans and found that the condition of one eye influenced the other covered eye of the same animal.

In 1932 Parker suggested the possibility of a hormonal agent in retinal pigment migration, a factor already hinted at by Welsh (1930*b*) and by Bennitt. This field of research was suggested to me by Professor G. H. Parker, to whom I wish to express my thanks and obligations for many kindnesses. The specific problem undertaken was to investigate the action of the crustacean eye-stalk hormone on the retinal pigments.

Material and Methods

The extracts of the eye-stalks of the various animals used in these experiments were prepared in essentially the same manner. A number of stalks were excised at their proximal ends and were triturated by means of a glass rod in a small Syracuse dish, the bottom of which had been previously roughened with powdered carborundum. The solvent was then added to this crushed tissue. Sea water was first used for this purpose, but was later abandoned in favor of amphibian Ringer's solution, since the latter was more constant in composition, being free from any dissolved foreign substances that might have occurred in the sea water, and could be used as a standard control substance.

After this preliminary treatment, the mixture was stirred for two minutes, as much of the coarse detritus separated off as possible, and the suspension of tissue in solvent drawn up into a hypodermic syringe. The extract was not filtered because of the small quantities prepared at one time. Control extracts of other tissues of the animals were prepared in the same way, or ordinary Ringer's solution with no tissue was used.

In those experiments where the extract was to be prepared from eyes adapted to darkness, use was made of two adjoining dark-rooms. The specimens were kept in one dark-room and were removed singly to the second room where, with the aid of a photographic safelight (Brownie Series No. 1) both eyes were excised and immediately crushed. With practice this operation could be performed within 30 seconds. It seems reasonable to assume that this short period of exposure to dim light would not induce in the eye the formation of a substance which might affect the retinal pigment. When a sufficient number of eye-stalks had thus been accumulated and crushed, the solvent was added and the process of extraction continued as above.

Injections of these extracts were made into the appropriate animals. In the first tests where extracts were injected into light-adapted shrimps little difficulty was encountered, but in later experiments the necessity of injecting in the dark-room into dark-adapted specimens presented certain technical difficulties. The initial results, disconcerting in their negativeness, were attributable to such factors as difficulty in capturing and removing an animal from the aquarium, penetration of the hypodermic needle too far into the shrimp with the result that the injected fluid entered the gill space, and injection of too large quantities of extract thereby causing the death of the specimen. With progress of the work, however, a satisfactory technique was evolved, which is considered of sufficient value to be described here in detail.

Before starting the experimental animals adapting to darkness, each shrimp was placed into a 600 cc. beaker containing about 300 cc. of sea water. The beakers were then transported to the dark-room and arranged in linear groups of five. Ten to twenty-five animals could thus be used conveniently for a single experiment. After an interval sufficient to assure dark-adaptation the specimens were ready for injection.

Parker (1897) found that the time required for complete outward migration of the distal pigment cells in adaptation to darkness was from 105 to 120 minutes, while Welsh's (1930*a*) observations indicate that the time for this migration is only slightly less, from 90 to 120 minutes. In the early part of this study shrimps were left in the dark-room usually overnight, but this was later shortened to a period of 5 hours. This interval also suffices for complete dark-adaptation of the proximal and reflecting pigments. Adaptation to light was obtained by keeping specimens in a white porcelain dish which was illuminated from above by an electric lamp.

Upon entering the dark-room, the first animal in the series was removed from its beaker and held in the left hand. After orienting the specimen with its dorsal side uppermost, the tip of the hypodermic syringe, which was held in the right hand, was inserted between two of the abdominal tergites. As soon as the needle was felt entering, it was twisted downward and slipped forward a fraction of an inch, so as to enter the abdominal musculature obliquely. The plunger was then tapped gently downward about half a dozen times, the needle withdrawn, and the shrimp replaced in its beaker. Immediate exit was made from the dark-room to record the time and amount of extract injected. This process was then repeated for the entire series of animals in an experiment. With a little practice, manipulation of the hypodermic plunger could be well controlled, with the consequence that, in the several hundreds of specimens used in this study, amounts of extract ranging from 0.01 cc. to 0.05 cc. were injected. Occasional difficulty was encountered when an animal jerked while injection was being made. In some such cases the needle might emerge and part of the extract would be lost. All cases of this sort where there was any doubt as to whether the extract had entered or remained in the specimen were noted. It may be added, parenthetically, that there could be no absolute certainty that the complete volume of injected extract remained within the animal; a small part might have been expelled by the body movements of the shrimp, but it is hoped that this possible error was reduced to a minimum by injecting obliquely forward.

In order to make readings of the position of the pigment in the eyes of the experimental specimens, two methods were used. In one (Welsh, 1930*a*), where the experimental animal was adapted to light, observations were made chiefly on the living shrimp. To this end the bottom of a Petri dish was covered with a layer of soft paraffin from which a block about one-half inch square was cut, exposing the glass bottom of the dish. A shrimp was fastened down by means of stationer's staples so that its eye-stalks projected over this opening and the dish was then filled with sea water sufficiently to cover the specimen. By placing the vessel on the stage of a microscope, the position of the distal retinal pigment could be observed and recorded by means of a micrometer eye-piece. After an injection of eye-stalk extract, the animal could again be fastened down and the position of the pigment measured at suitable intervals.

In the second method where the specimens were adapted to darkness before injection of the stalk extract was made, it was necessary to fix the eyes before any readings could be taken of the position of the pigment. This was done by killing animals at varying intervals after injection by immersion in hot water (80°–90° C.) for 10 to 15 seconds. Following this preliminary fixation, the eye-stalks were excised, dehydrated, and cleared, either in oil of cedarwood or in beechwood creosote. The position of the distal pigment in the cleared eyes could then be measured. It was necessary to embed and section the eyes to observe the proximal and reflecting pigments. This was facilitated by first washing the cleared eye-stalks with ether-alcohol solution and then embedding doubly in celloidin and in paraffin. Sections were cut at 10 micra, some being subsequently stained with Delafield's hematoxylin and eosin, and others being mounted unstained.

The animals used in this study were all crustaceans of the order Decapoda. The common marine shrimp, *Palæmonetes vulgaris,* was chosen as the test organism and, for a number of reasons, proved to be very favorable material. First, it is a commonly occurring form that is easily obtained in sufficient quantities. These specimens were readily secured in Boston from a dealer in live bait. During the summer months at Woods Hole they were caught by sweeping a dip-net along pilings and rocks covered with the alga, *Fucus.* Secondly, the mechanics and time periods for retinal pigment migration are well known through the excellent studies of Parker and of Welsh. Thirdly, the test shrimp, *Palæmonetes,* does not experience the retinal condition of persisting diurnal rhythm (personal communication from Dr. Welsh) which has been found occurring in a number of crustaceans (Welsh, 1930*b*, 1935; Bennitt, 1932*b*).

Animals whose eye-stalks were tested for the presence of a hormone that affected retinal pigment were: *Palæmonetes vulgaris,* the common shrimp; *Uca pugilator,* the fiddler crab; *Libinia dubia,* the spider crab; *Cancer irroratus,* the rock crab; *Carcinides mænas,* the green crab; and *Callinectes sapidus,* the blue crab. The results of this investigation are considered below under separate headings.

STRUCTURE OF THE EYE OF PALÆMONETES

For a detailed description of the finer structure of the eye and an account of the mechanics of pigment migration, the reader is referred to the papers of Parker (1891, 1897) and of Welsh (1930*a*, 1932). For this paper a description of the three types of pigment cells and their relation to the eye as a whole is deemed sufficient.

The eye of *Palæmonetes* is a compound structure consisting of ommatidial units. Composing or concerned with each ommatidium (Fig. 1) are the following structures: cornea (*C*), cone, rhabdome (*RH*), fenestrated basement membrane (*BM*), and the three types of pigment cells, the latter enriched by a varied terminology, but for purposes of simplicity and greater descriptiveness referred to here as distal (*DP*), proximal (*PP*), and reflecting (*RP*) pigment cells.

In each ommatidium there are two distal pigment cells which form a collar around the cone. These cells possess distal processes which extend to the cornea, and proximal processes which appear to be continuations of the proximal pigment cells.

There are eight proximal cells, one of which is rudimentary, to each ommatidium, all surrounding the rhabdome. Proximally beyond the rhabdome these cells become attenuated, pass through the fenestrated basement membrane, and, as retinal nerve fibers, connect with the first optic ganglion. These proximal cells appear to be the only retinal cells to have an anatomical connection with the central nervous system.

The reflecting pigment cells are located near the basement membrane. Processes from them may extend proximally through the basement membrane as far as the first optic ganglion, while distal processes may reach outward to the tip of the distal retinal cells where some of the reflecting pigment is accumulated in a small cap. The number of reflecting cells is difficult to determine unless sections are treated by special histological methods to remove the pigment and stain the nuclei, but Parker (1897) believes that there are no more than one or two of these cells for each ommatidium.

Black pigment granules are contained in both the distal and the proximal retinal cells. In the reflecting cells the pigment is of a different sort. When this pigment is viewed through the microscope by transmitted light, it appears yellowish or greenish brown and is barely distinguishable from the black pigment of the proximal and distal cells; examination of the same section by dark-field illumination, however, shows the reflecting pigment as a gleaming white granular mass. The pigment itself is probably guanin, though, as far as I am aware, few studies of the chemical nature of this white pigment have been made among the

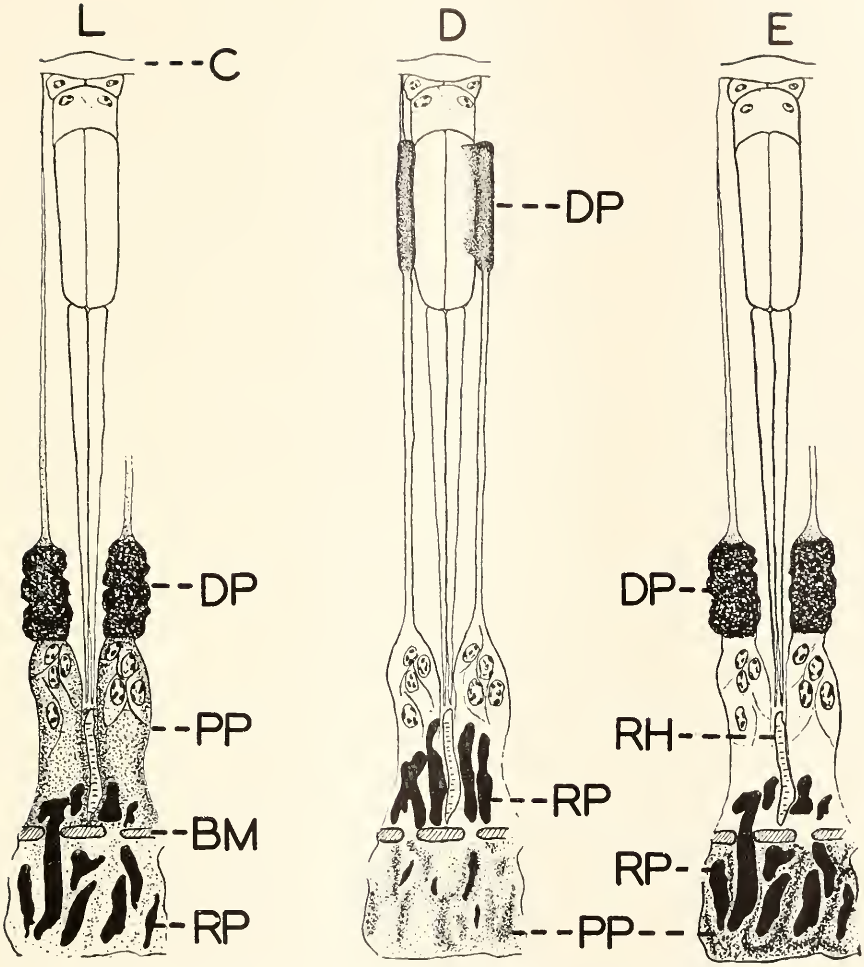


FIG. 1. Ommatidia from the eyes of *Palæmonetes vulgaris,* showing the general structure and the position of the three pigments under various conditions. *L,* from an eye in the light condition; *D,* from a dark-adapted eye; *E,* from an experimental animal which, after being adapted to darkness, was injected with stalk extract prepared from the eyes of light-adapted specimens. *C,* cornea; *DP,* distal pigment; *PP,* proximal pigment; *BM,* basement membrane; *RP,* reflecting pigment; *RH,* rhabdome.

invertebrates and none among the crustaceans. Guanin is commonly found occurring in crystalline condition in the integument of lower vertebrates (Ewald and Krukenberg, 1882, 1883). It is thought by some investigators to be formed from alimentary nucleoproteids and by others to be formed endogenously from nucleoproteids of the body tissues (Millot, 1922, 1923*a*, 1923*b*).

NORMAL PHOTOMECHANICAL CHANGES IN THE EYE OF PALÆMONETES

The positions of the retinal pigment cells characteristic for the conditions in darkness and in light are shown in Fig. 1. The distal pigment cells in eyes which have become fully adapted to darkness have migrated distally so that they terminate approximately at the outward level of the cones. In this position, according to Exner (1891), lateral rays of light might be admitted through one cone and then be allowed to diffuse to the receptors of adjacent ommatidia, thereby increasing the efficiency of the eye.

In the light condition, the distal pigment cells have migrated proximally a distance of 150–200 micra, and have the form described by Herrick (1891) of a "plaited black ribbon." Parker (1897) regarded this appearance as abnormal, due to a prolonged stay in the dark before exposure to light, but Welsh (1930*a*) has found this to be a normal occurrence. I find this same condition to be typical in my preparations of light-adapted eyes. In this position the distal pigment cells prevent overstimulation of the rhabdome by excluding lateral rays and by admitting only those axial light-rays which enter the rhabdome by way of the cone.

The proximal pigment may be considered as moving in directions opposite to that taken by the distal pigment in response to light and to darkness. In the light, the pigment granules are found in a distal position above the basement membrane, while in a dark-adapted eye the pigment has migrated proximally below the basement membrane. The means by which the migration of the proximal pigment occurs has not been studied, but it has been assumed by Parker (1897), Mossler (1915), and Bennitt (1924) to result from some form of protoplasmic movement.

Exner (1891) first called the layer of reflecting pigment the tapetum. Its function appears to be the reflection of light from within the eye so that in darkness or in dim light, rays, which have entered the eye and have passed through the rhabdome, may be reflected back again into the rhabdome or even out of the eye. Stimulation may thus be accomplished by repeated application of weak light to the rhabdome, where ordinarily the light would have been of insufficient intensity to have any retinal effect. The glow which emanates from the eyes of many crustaceans which have been kept for some time in the dark is due to the reflection of light from the eye by the reflecting pigment.

It was believed for a time that the reflecting pigment did not change its position under various conditions of light, and from the common understanding that this pigment acted solely as a reflector no photomechanical change was considered necessary, so long as the proximal pigment moved to cover or uncover the tapetum. But Parker (1897) thought that the reflecting pigment in *Palæmonetes* underwent positional changes in adaptation to light and to darkness. This was supported by reports of Trojan (1913) and of Mossler (1915) on *Palæmon*, and confirmed and extended by Welsh on *Palæmonetes* and on *Macrobrachium* (1932). In the dark-adapted eye the greater part of the reflecting pigment lies above the basement membrane (Figs. 1, 5, 9, and 10), while in a fully light-adapted retina much of it, except for a small portion, lies in processes below the basement membrane (Figs. 1, 6, 11, 12). Little is known about the mechanics of migration of this pigment.

Palæmonetes vulgaris

Four groups of tests involving the use of eye-stalk extract from this animal were carried out. In the first two groups, observations were made on living specimens in the manner already described above; in the last two, the eyes of the test shrimps were fixed, dehydrated, and cleared before measuring the migration of the distal retinal pigment.

Table I

Proximal migration of the distal retinal pigment cells in a series of dark-adapted test *Palæmonetes* as a result of injecting individuals with 0.02 to 0.04 cc. of an extract of 60 eyes of light-adapted *Palæmonetes* in 1.0 cc. of sea water. Specimens were killed at various intervals after injection. The position of the pigment is given in ocular micrometer units (1 unit being equal to 16.2 micra) and was measured from the surface of the cornea to the distal margin of the pigment cells.

Minutes after injection	Each eye	
0	4.0	3.5
0	3.0	3.5
0	5.0	4.5
5	5.0	6.0
10	6.0	6.0
15	9.0	6.5
18	12.0	10.5
20	8.0	12.0
21	11.0	11.5
22	15.0	17.0
25	13.0	11.0
28	14.0	17.0
30	15.0	15.0
32	17.0	16.0
34	13.0	12.0
36	19.0	19.0
38	16.0	16.5
45	15.0	20.0
50	19.0	18.0
55	18.0	19.5

In an attempt to determine whether the crustacean eye-stalk hormone would cause an elongation of the distal pigment cells in light-adapted shrimps, suitable specimens were injected with two kinds of extracts. Some received extract prepared from the stalks of shrimps that had been in the dark-room overnight, while others were treated with stalk extract from the eyes of animals which had been adapted to a black background. In neither of these cases was there any significant change in the position of the distal pigment cells (Kleinholz, 1934).

In the third group of tests, the entire procedure was reversed in order to see whether the eye-stalk hormone would induce a contraction of the distal retinal pigment similar to that produced on the body pigment. Stalk extracts were prepared from the eyes of light-adapted specimens and were injected, in the dark, into test shrimps which had been adapting to darkness overnight. An extract of 60 stalks in 1.0 cc. of sea water was prepared and injected in doses ranging from 0.02–0.04 cc. into the animals of a series. After the preliminary fixing and clear-

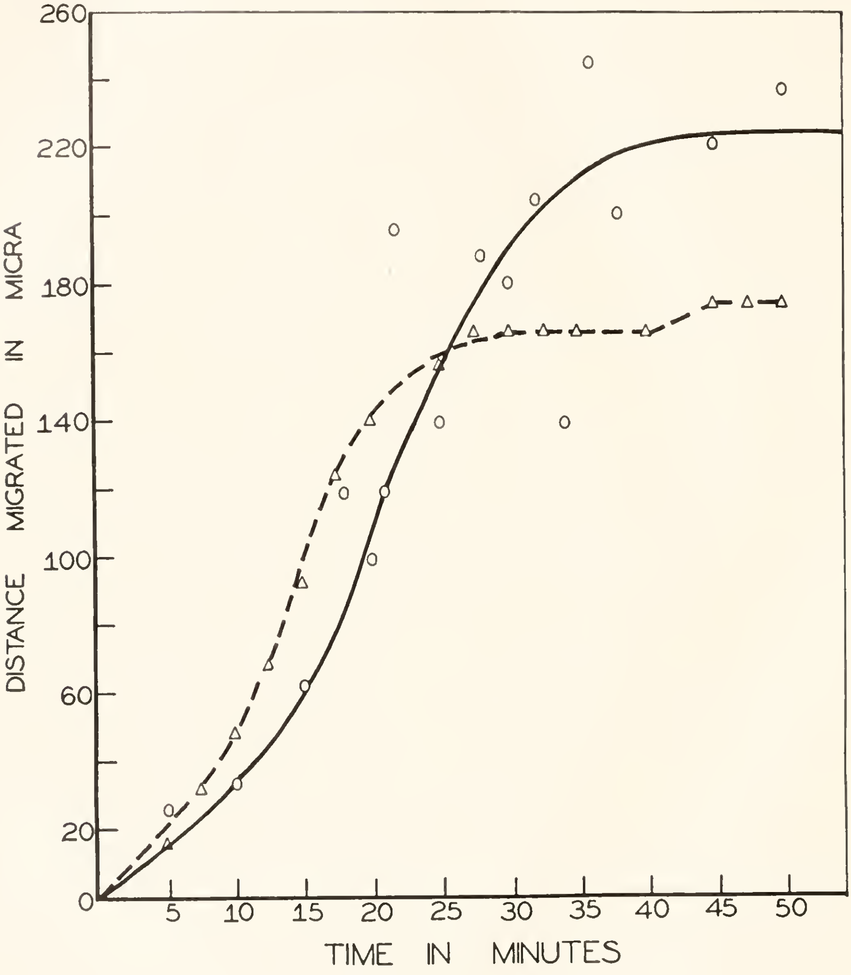


Fig. 2. Results of the measurements shown in Table I, plotted to show rate of migration of the distal pigment cells in a series of animals which have been injected, while adapted to darkness, with extract from the eye-stalks of light-adapted *Palæmonetes*. The broken curve represents Welsh's figures on the rate of migration of the distal pigment during adaptation to light.

ing of the stalks of the injected shrimps, the position of the distal retinal pigment was measured by means of a micrometer eye-piece. Proximal migration of this pigment had occurred, a maximum being reached in those animals killed from 30–45 minutes after injection.

The set of measurements for one such series is represented in Table I. The three uninjected controls were killed at intervals during the course of the experiment, one when injections into the test shrimps were about to be started, one at about the middle of the experimental period, and the third when the last injected specimen was fixed. Since the controls showed some variation in the position of the pigment, not only among themselves, but also between both eyes of the same animal, the average position of the distal pigment in all six eyes was taken as the " zero reading " for dark-adaptation.

The average measurements from both eyes of the test specimens of this series, when converted into micra and plotted against time after injection, give the curve (solid line) shown in Fig. 2. The broken-line curve in this figure is a transcription of Welsh's measurements on the rate of migration of the distal pigment cells during the course of adaptation to light. The general similarity of the two curves is at once evident. The rate of change is slow at first, but after 10 minutes is increasing rapidly, being 10 micra per minute for Welsh's curve, and about 6.5 micra per minute for mine. Welsh's figures show that the rate of migration begins to decline after about thirty minutes when the migration has neared its maximum, but that there is a secondary movement when the distal pigment cells contract into the plaited form observed by Herrick (1891). Since my measurements were made on a number of animals and are consequently subject to more variation than Welsh's consecutive readings on the same individual, I have not felt justified in indicating this secondary movement graphically, although from a study of my sectioned material it is known to occur.

The difference in rate of migration between the two curves invites interesting speculation. If we assume that in the dark no effective hormone is being released into the blood stream and that with light stimulation such a hormone is secreted until the pigment has contracted, we may expect that constant stimulation of the eye by light will result in a continuous secretion of the hormone to maintain the pigment in a state of contraction. Since my test specimens, however, were kept in the dark-room throughout the course of the experiment, the only hormone present within the body was that injected into the shrimps. The dosage and concentration used makes this equivalent to 1.5 light-adapted eyestalks injected into each individual. The experimentally injected animals thus not only had a smaller amount of hormone than light-adapting shrimps, but were also lacking a continued secretion which would occur during the process of adaptation to light. At this time, however, not very much importance can be attached to this aspect of the problem. More exact quantitative treatment must be delayed until the crustacean eye-stalk secretion can be isolated or prepared in purer form.

The scatter and the greater amount of migration obtained in my curve is due, I think, to variations in size of individual eyes and to the fact that my specimens were probably slightly larger than those used by Welsh.

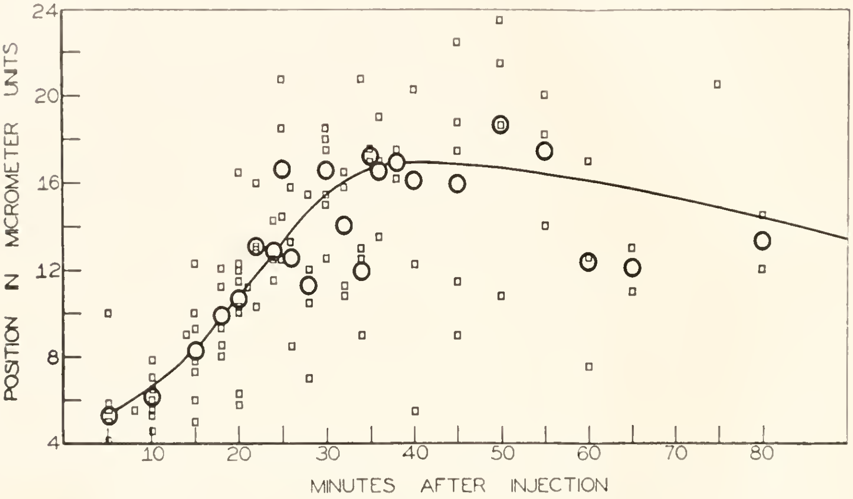


FIG. 3. Graph showing the change in position of the distal pigment in the eyes of dark-adapted *Palæmonetes* at varying intervals after injection with light-adapted eye-stalk extract. Each square represents the average of the positions in both eyes of a single animal, and the larger circles represent the averages of all eyes measured at similar intervals after injection. The wide scatter shows the inadequacy of this method for quantitative measurements.

Any attempt at an exact quantitative determination of rate of migration of the distal pigment, made by killing individuals at varying intervals, is necessarily unsatisfactory. For rough estimates, these methods, used by Parker (1897), Mossler (1915), and Welsh (1930*a*), indicate the time required for complete adaptation to light or to darkness. But even so there is a considerable degree of deviation. Welsh reports, "In some eyes the distal pigment cells would have reached the extreme position characteristic for the light in thirty minutes, and in other eyes this would not be reached until sixty or seventy minutes in the light."

Mossler (1915) attempted to reduce this error by taking measurements on a number of eyes after each period of exposure to light and then averaging the results. But this too was not very satisfactory. I have attempted a similar though more extensive method, using measurements made on about 200 eyes from dark-adapted *Palæmonetes* which had been injected with extracts of light-adapted stalks. The results are shown in Fig. 3. In this graph each square represents the average of the position of the distal pigment in both eyes of an experimental animal; the large circles represent the averages for all specimens killed at the same periods after injection. Except for about half a dozen specimens which showed little or no response to treatment with the eye-stalk extract, possibly because of inadequate injections, practically all of the other experimental animals showed some degree of distal pigment migration, although here too there is some discrepancy, even in animals killed at similar times after injection. These variations are reasonably attributable to such factors as individual differences in the size of the eyes and in the physiological conditions of the animals. Welsh (1930*a*) came to the conclusion that no really accurate measurement of the rate of migration of the distal pigment could be made except by studying this process in single individuals. Similarly, it is my opinion that any further quantitative studies of hormonal effects on this process will need to be made by a method identical with or similar to that finally used by Welsh.

When sections of eyes from experimental animals in this third group of tests were examined histologically, the proximal migration of the distal pigment was confirmed. The proximal pigment was apparently unaffected by the stalk extract, since it remained in the position typical for darkness. The reflecting pigment, however, was observed to have migrated proximally into the light condition. In an earlier paper, Welsh (1935) mentioned some of my unpublished results where the eye-stalk hormone was reported to have no effect on the reflecting pigment. At that time I had examined the first of my preparations only superficially and was misled by the difficulty of distinguishing, by transmitted light, between the pigment in the proximal and in the reflecting cells. Subsequent examination of the same and additional material by means of dark-field illumination showed that after injection of stalk extract the greater part of the reflecting pigment had moved below the basement membrane (Figs. 1, 7, 8, 13, 14).

Two control experiments were performed for this third group of tests. Whenever a series of *Palæmonetes* was put into the dark-room in preparation for injection with stalk extract, several primary control specimens were included along with it. These primary controls were uninjected and were fixed at intervals during the course of each series. The distal pigment in the eyes of such controls showed variations of

1–3 micrometer units (16–48 micra). Three series of secondary controls consisted of specimens which were first adapted to darkness and then injected either with sea water, cold-blooded Ringer's solution, or sea-water extract of shrimp's abdominal musculature. A total of 47 specimens were treated as secondary controls and in none of these was there any momentous change in the position of the distal pigment cells. The slight variation noted in the primary controls was also evident here, but this was undoubtedly due to differences in the eyes of individuals and has no significance for these experiments.

In the fourth group of tests, shrimps were adapted to darkness as before, but the stalk extract was prepared from *Palæmonetes* which had been adapted to darkness. The method of preparing these dark-room extracts has been described above. Concentrations equivalent to 60 stalks in 1.0 cc. of amphibian Ringer's solution were injected in small quantities (0.02–0.04 cc.) into 41 dark-adapted test animals. The specimens were killed at an average time of 33 minutes after injection and the position of the distal pigment measured. A partial migration of the distal pigment towards the condition characteristic for light occurred, but this movement was not as marked as that resulting from the injection of extracts prepared from light-adapted stalks. For purposes of comparison the average position of the distal pigment in the eyes of this group are represented graphically with that of the third group where the same concentration of extract was injected into a nearly equal number of specimens (Fig. 4). Extracts from light-adapted eyes are seen to be almost twice as potent as those prepared from dark-adapted eyes. The probable significance of this difference will be discussed below.

Cancer irroratus

In examining the effects of stalk extracts from the eyes of various crustaceans on the retinal pigment of the test animal, *Palæmonetes*, the chief interest centered on the response of the distal pigment cells which were most easily observable, but where possible, the fixed eyes of the test shrimps were sectioned to study also the reactions of the proximal and reflecting pigments.

Since it became apparent from the early work with *Palæmonetes* extract that no reliable measurements could be obtained for the rate of response of the distal pigment to stalk extracts by the method used, it was decided to abandon this procedure, and, by a roughly quantitative method, attempt to study the effects on an " average " test specimen. Shrimps which survived treatment with a particular extract were killed at an interval of 30–40 minutes after injection. The average time the extract was allowed to act before killing the test animals and the average

Table II

Average time extract was allowed to act before killing animals and average position of distal pigment

Source of eyestalks	Number of animals injected	Number died	Average time killed after the injection	Number of eyes measured		Position of distal pigment		Average position of pigment	
				Injected	Control	Injected	Control	Injected	Control
			min.						
Cancer irroratus	46	0	33.8	91	16	24,121	1,360	265.0	85.0
Libinia dubia	46	4	35.0	84	20	28,204	1,636	335.7	81.8
Uca pugilator	31	2	36.4	58	26	14,337	2,300	247.2	88.3
Callinectes sapidus	66	13	31.4	106	18	10,935	1,425	102.8	79.2
Palæmonetes vulgaris (light-adapted)	30	0	32.6	60	14	14,742	810	245.7	57.8
Palæmonetes vulgaris (dark-adapted)	41	6	32.5	70	18	10,465	1,409	149.5	78.3
Carcinides mænas	34	6	38.0	56	19	11,485	1,506	205.1	79.3

position of the distal pigment for all the eyes studied in a series were then calculated and tabulated (Table II). The measurements obtained from the eyes of primary controls were treated in the same fashion. A number of secondary controls, consisting of test *Palæmonetes* into which various tissue extracts had been injected, were also studied, but these observations were not included in the tabulated results.

In this first series, 46 specimens of *Palæmonetes,* adapted to darkness, were injected with small quantities (ranging from 0.02 to 0.04 cc.) of an extract of 10 light-adapted eye-stalks of *Cancer irroratus* in 0.8 cc. of amphibian Ringer's solution. The test animals were killed about 35 minutes after injection and the position of the distal pigment measured. The range of response was variable, as might have been expected from the earlier work. The lowest value (in one eye) was about 194 micra and the highest value (in one eye of another specimen) was

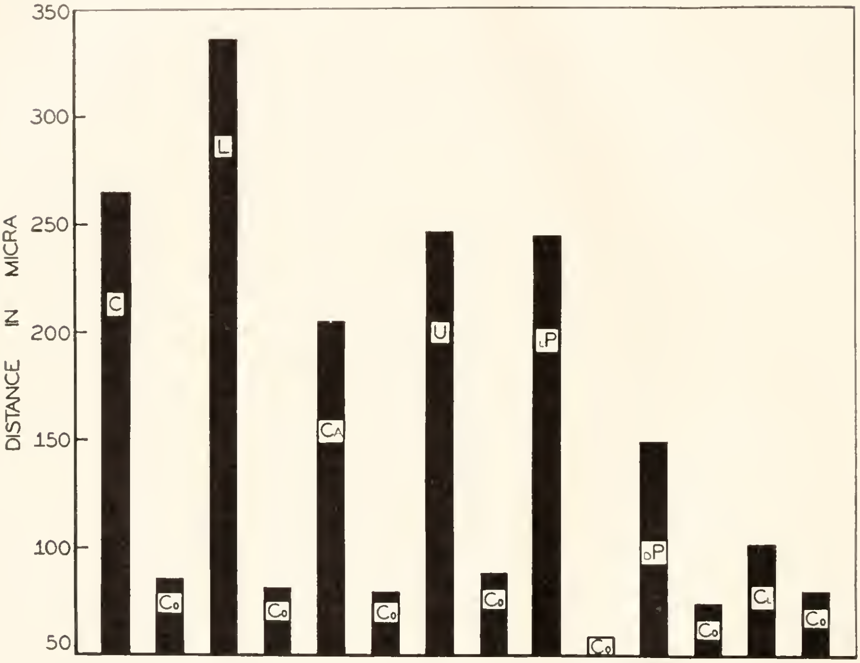
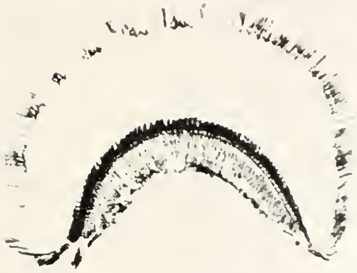


FIG. 4. Graphic representation of the position of the distal pigment in "average" test specimens as a result of injecting extracts from the eyes of various decapod crustaceans. *Co*, control uninjected specimens; *C*, extract from eye-stalks of *Cancer irroratus*; *L*, extract from eyes of *Libinia dubia*; *CA*, stalk extract from *Carcinides mœnas*; *U*, extract from *Uca pugilator*; *LP*, extracts from eyes of light-adapted *Palæmonetes vulgaris*; *DP*, extracts from darkness-adapted eyes of *Palæmonetes vulgaris*; *CL*, extracts from eyes of *Callinectes sapidus*.



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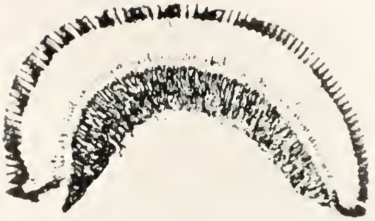

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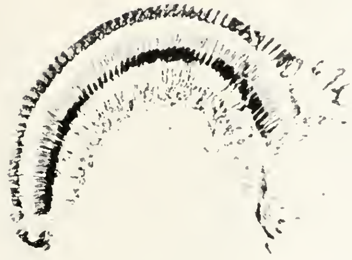

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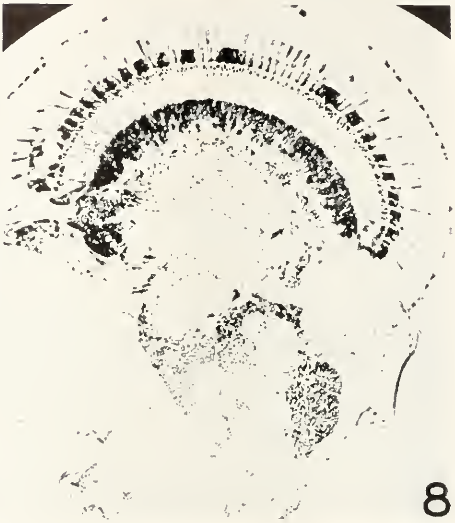

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Explanation of Plate I

Fig. 5. Longitudinal section of a normal retina of *Palæmonetes vulgaris* in the "dark" condition. Only portions of the distal pigment cells show, due to the plane of sectioning. The reflecting pigment appears as a dense black mass above the basement membrane which is clearly visible. Below the basement membrane is the more dispersed proximal pigment. At the very bottom of the section are traces of reflecting pigment which apparently never migrates completely above the basement membrane.

Fig. 6. Longitudinal section of a normal light-adapted retina of *Palæmonetes*. The distal pigment has migrated proximally. Immediately below the distal pigment is the diffuse proximal pigment which has migrated above the basement membrane. Below the proximal pigment is the mass of reflecting pigment from which many processes are seen extending below the basement membrane.

Fig. 7. Longitudinal section of a dark-adapted *Palæmonetes* retina. This specimen was injected, in the dark, with 0.02 cc. of an extract of 20 light-adapted *Palæmonetes* eye-stalks in 0.4 cc. of sea water. The retina was fixed 45 minutes after injection. The distal pigment has migrated proximally. The proximal pigment has remained below the basement membrane, while nearly all of the reflecting pigment has moved proximally below the basement membrane.

Fig. 8. Longitudinal section of the eye-stalk of a dark-adapted *Palæmonetes*. This specimen was injected, in the dark, with 0.03 cc. of extract of 4 light-adapted *Libinia dubia* eye-stalks in 0.4 cc. of amphibian Ringer's solution, and was killed 35 minutes after injection. Stained with Delafield's hematoxylin and eosin. The position of the three pigments is the same as in Fig. 7.

372 micra; both of these specimens were killed at the same interval after injection of almost equal amounts of the extract. The average positions of the distal pigment for the 46 experimental (265 micra) and the 16 primary control shrimps (85 micra) are shown graphically in Fig. 4. Six secondary controls were injected with 0.02–0.05 cc. of an extract of cheliped muscle in Ringer's solution. The average time specimens were killed after injection was 30.5 minutes and the average position of the distal pigment was 69.2 micra.

The eyes of two of the test animals were embedded and sectioned. The proximal pigment was seen to have remained in the position characteristic for darkness, below the basement membrane, but the reflecting pigment had, for the most part, moved below the basement membrane into a position typical for light-adapted eyes.

Libinia dubia

In this experiment 46 test *Palæmonetes* were injected with 0.01 cc. to 0.04 cc. of an extract of light-adapted eye-stalks from *Libinia dubia*. The concentration used, equivalent to 10 stalks in 1.0 cc. of Ringer's solution, was well tolerated by the shrimps, only 4 of them dying during the course of the experiment. Of the 42 surviving animals, two failed to show any response of the distal pigment, but all others showed a very marked contraction of the distal cells, in several specimens the position being measured at 437 micra from the margin of the cornea. The average position for the 42 surviving *Palæmonetes*, including the two which showed no response at all, was 335 micra, and that for the primary controls was 82 micra. Five secondary controls were injected with extract prepared from the maxillipeds of *Libinia*. The average time during which the extract was allowed to act was 32 minutes, and the average position of the distal pigment at the end of this time was 76 micra.

EXPLANATION OF PLATE II

These photographs were taken by means of a Zeiss cardioid condenser.

FIG. 9. The same section shown in Fig. 5. This print has been retouched to indicate the expanded position of the distal pigment cells, made evident by the small caps of reflecting pigment at the distal ends of these cells. The main mass of the reflecting pigment lies above the basement membrane.

FIG. 10. A high-power detail of a portion of Fig. 9, showing the pigment above the basement membrane and the small amount at the proximal limit of the retina.

FIG. 11. The same section as shown in Fig. 6. The contracted condition of the distal pigment cells is indicated by the caps of reflecting pigment on the cells.

FIG. 12. A portion of Fig. 11 in high-power view. The reflecting pigment is seen extending proximally in processes below the basement membrane.

FIG. 13. The same as the section in Fig. 7.

FIG. 14. An enlargement of a portion of Fig. 13, showing the large number of processes containing reflecting pigment, due to a proximal migration induced by injection of eye-stalk extract.

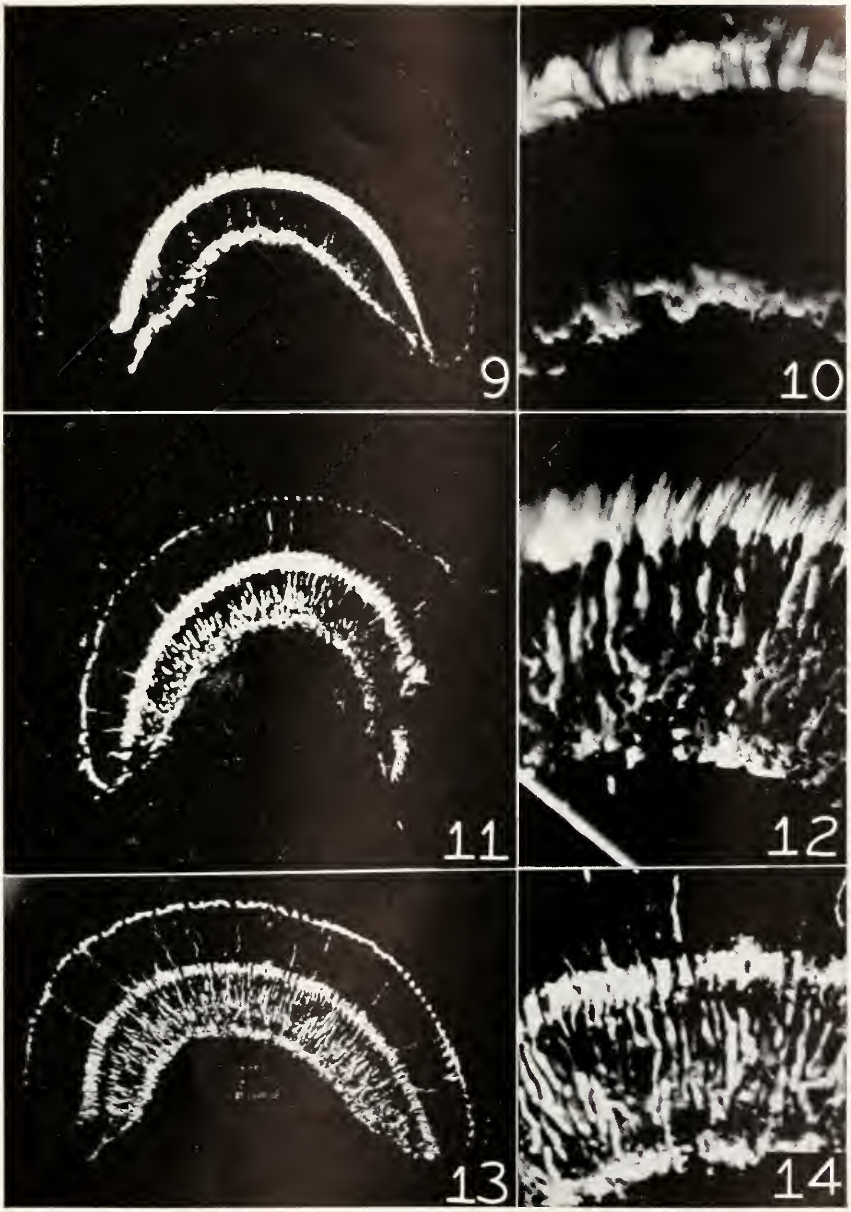
9
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Plate II

Sections of the eyes of three of the test *Palæmonetes* showed the proximal pigment persisting in the dark condition, but the reflecting pigment migrated into the light condition (Fig. 8).

Uca pugilator

Thirty-one test shrimps, which had been adapted to darkness, were injected with 0.02 cc. to 0.04 cc. of an extract of *Uca* eye-stalks, a concentration of 15 stalks to 1.0 cc. of Ringer's solution being used. Two of the test animals died during the course of the experiment, and only one of the 29 surviving *Palæmonetes* failed to show any change in position of the distal pigment; this exception was one that had been designated as doubtfully injected. In three eyes the position showing a small amount of migration was as low as 128 micra, but in the remaining eyes the range was up to a maximum of 356 micra. The average position for the test specimens was 247 micra, for the primary controls it was 88 micra, and for 5 secondary controls (cheliped muscle in Ringer's solution) the position was 68.0 micra.

The eyes of two of the *Palæmonetes* injected with extract from the stalks of *Uca* were sectioned. The proximal pigment was in the position characteristic for darkness, while the reflecting pigment showed a slight migration into the position characteristic for the light condition.

Carcinides mænas

In a preliminary experiment, 12 test *Palæmonetes* were injected with small amounts of *Carcinides* eye-stalk extract, in a concentration of 10 stalks per 1.0 cc. of saline solvent. This proved to be too concentrated, resulting in the death of 5 of the injected shrimps. With a slightly diluted extract (equivalent to a bit less than 8 stalks in 1.0 cc. of amphibian Ringer's solution) the survival was much better, only one of the test specimens succumbing to the injection. Five of the injected animals showed only very slight evidence of change in position of the distal pigment (the reading being at about 130 micra), while the remaining shrimps showed responses that were marked but slightly lower than those obtained previously with extracts from other crustaceans (Fig. 4).

The average position of the distal pigment in 26 surviving test specimens was 205 micra, that for 10 uninjected primary controls was 79.3 micra, and for 6 secondary controls injected with extract of cheliped muscle, the position was 76.5 micra.

No eyes were sectioned for observation of the proximal and reflecting pigments.

Callinectes sapidus

In early tests, the injection of extract prepared from the eye-stalks of *Callinectes*, in a concentration of 2 stalks to 1.0 cc. of Ringer's solution, produced a very slight, almost doubtful response of the distal pigment. In an attempt to increase the magnitude of the reaction, extracts of higher concentration (5 stalks to 1.0 cc. of solvent) were prepared and injected. Unfortunately, in these cases the crude extract proved to be very toxic, killing many of the test *Palæmonetes*. Those animals which did survive the injection did not show any increased response over those treated with the more dilute extract. The average position of the distal pigment for 53 surviving test specimens was 102.8 micra, and that for 9 primary controls was 79.2 micra. No secondary controls were carried for this series, and none of the eyes were sectioned for observation of the other retinal pigments.

The results obtained by using extracts prepared from eye-stalks of *Callinectes* are difficult to understand. Perkins and Kropp (1932) and Hanström (1935) found that stalk extracts prepared from this crustacean were effective in concentrating the body pigment of *Palæmonetes;* the potency of my extracts was tested by injection into dark *Palæmonetes* and the typical contraction of the body pigment was obtained. The response of the shrimp's retinal pigment to this extract is so slight as to be almost within the limit of experimental error. Two possibilities suggest themselves in explanation of this situation. There may be a difference in concentration threshold between the retinal pigment and the body pigment, and it may be that my extracts were not sufficiently purified (by filtering) to allow the use of more concentrated preparations which would not prove toxic. Or else, it is possible that crustaceans possess several pigmentary hormones, and that in *Callinectes* the hormone affecting the retinal pigment is lacking. This point requires further experimental investigation.

DISCUSSION

In studies of processes under hormonal control two general methods of procedure are open to the investigator. One is to remove the tissue thought to secrete the active agent, and the other is to inject extracts of the organs suspected of endocrine function.

Unfortunately, little use can as yet be made of the first method in studies of the crustacean pigmentary hormones, because there is a scarcity of information about the specific tissue within the eye-stalk which is involved in such activity. Only recently have there been any morphological studies of the crustacean eye that have revealed the presence of structures with a possible hormonal function. This has been reported by Hanström (1931, 1934) and one of his students, Sjögren (1934). These investigators found two different tissues, an X-organ and a "blood gland," which they think may play a part in the control of crustacean color changes. Professor Hanström has very kindly allowed me to read a preliminary paper of his which is in press at the time this paper is being written. He reports the findings of an experimental study made in an attempt to find a probable connection between the blood gland and the chromatophore activator. The results of a similar study on *Uca* have already been published (Carlson, 1935). According to Carlson, the middle third of the eye-stalk of *Uca* contains the active chromatophorotropic principle. Histological examination shows that the middle third of the stalk contains the well-developed blood gland, whereas the X-organ is either very small or completely absent. "In *Pagurus* . . . the blood gland . . . extends through both proximal and middle thirds of the eyestalk. The X-organ is situated in the proximal third, which thus contains part of the blood gland and the whole X-organ. Since the middle third of the eyestalk of *Pagurus,* that contains only part (the greater part, however) of the blood gland, concentrates the red and yellow pigment at least as strongly as the proximal third does, this fact, in connection with those mentioned above, speaks in favor of the view that the blood gland is the real source of the pigment concentrating substance in the decapod crustaceans" (Hanström, 1935). As Hanström recognizes, these facts do not exclude the X-organ from some part in the control of crustacean color changes. A more detailed knowledge of the rôles played by these two structures must await results from experimental removal or destruction of the endocrine tissues.

The second of these methods, used in this study, demonstrates fairly convincingly, that some endocrine organ within the eye-stalk is effective in causing two of the retinal pigments to migrate from a position that is characteristic for adaptation to darkness into a second position which is typical for an eye adapted to light.

It is interesting to note, in connection with this result, that a greater amount of migration of the distal pigment is obtained with the use of extracts prepared from eyes that were adapted to light, than with the use of extracts of an equal number of eyes which had been adapted to darkness. The explanation of this probably lies in the manner in which the eye-stalk hormone is synthesized and released by the animal. If the hormone is being formed continuously and stored until the time of release by light, acting as the stimulus, then there should be no difference in the potency of extracts prepared from light-adapted eyes and those prepared from eyes adapted to darkness. If, on the other hand, no hormone is being built up in darkness, the response which was obtained from extracts of eyes adapted to the dark-room might be attributable to residual amounts of the effective hormone present in the gland through previous activity in response to light. This possibility merits cytological investigation.

In 1900, Gamble and Keeble reported a case of persistent rhythmic change in the chromatophores of *Hippolyte varians* even though the conditions of light remained constant. Slome and Hogben (1929) found a similar case of diurnal rhythm persisting in the South African frog, *Xenopus lævis*, after the removal of the normal stimuli. This animal, while kept in constant darkness, shows the melanophores more contracted at midnight than at midday.

Welsh (1930*b*) was the first to report a similar condition for the retinal pigment of crustaceans. Although *Macrobrachium olfersii* and *Macrobrachium acanthurus*, both nocturnal animals, were kept under constant illumination, the *distal pigment* continued to show a diurnal movement, migrating distally at 6:00 P.M. and remaining in this position until 5:00 A.M. the following morning, when it returned to the proximal position characteristic for light-adaptation. The proximal pigment did not exhibit such persisting rhythm.

Welsh's observations were soon followed by a similar one reported by Bennitt (1932*b*), who found that a rhythm persisted in the *proximal pigment* of the crayfish, *Cambarus,* even though the animal was kept in constant darkness. Subsequent studies made by Welsh (1935) found this phenomenon occurring in a number of other crustaceans. In *Latreutes fucorum* and in *Leander tenuicornis* the rhythm occurs in the *reflecting pigment* (and to a slight extent, perhaps, in the distal pigment) which, during the night, remains above the basement membrane in spite of constant illumination. *Leander affinis* shows "a definite rhythm in the movements of the reflecting pigment and a partial migration of the distal pigment" occurring "daily whether the animals are kept in constant light or darkness" (Welsh). *Penæopsis goodei,* a distinctly nocturnal form, possesses a persistent rhythm of the *proximal pigment,* being similar to *Cambarus* in this respect.

The physiology of these activities in crustacean retinal pigment is as yet little understood. Welsh concluded from his experiments with *Macrobrachium* that the normal migrations of the distal pigment cells as well as their periodic movements under constant conditions of light were controlled directly by the blood and indirectly by the nervous system. The mechanism underlying this phenomenon of persisting rhythm has been too little studied to warrant any final conclusion. The fact that a persistent rhythm may exist for one kind of pigment in one species, and for a second or third kind of pigment in another species, that it may persist in constant darkness for one crustacean and in constant light for another, all add to the complexity of the situation. Are we to attribute these different rhythms to several different hormones, or is there only one hormone to which these various pigment cells respond differentially because, let us say, of threshold differences? In *Macrobrachium*, is the rhythm due to liberation of a hormone, or is it due to cessation of hormone secretion? Is the nervous system directly involved in rhythms of the proximal pigment? If endocrine glands are involved, are they in the position of intermediate agents, secretion being started or stopped by a rhythm in the nervous system, or do the glands themselves function in a rhythmical fashion? All of these possibilities must be considered and tested experimentally before we can proceed to an understanding of this intensely interesting phenomenon.

SUMMARY

1. Specimens of *Palæmonetes vulgaris* which had been exposed to light so that their retinal pigments were in positions characteristic for that state were injected with crustacean eye-stalk extract, in one series prepared from animals that had been in the dark-room overnight, and in a second series prepared from specimens that had been kept on a black background. There was no significant change in the position of the distal retinal pigment cells in either series.

2. When stalk extracts were prepared from light-adapted animals and were injected, in the dark, into *Palæmonetes* in which the retinal pigments were adapted to darkness, a proximal migration of the distal retinal pigment occurred towards the position found typically in the eyes of specimens adapted to light.

3. If the rate of distal pigment migration following experimental injection is plotted, a sigmoid curve is obtained similar to that found by Welsh for the normal rate of migration when a single individual adapts to light.

4. Histological study of the eyes of experimentally injected *Palæmonetes* shows that the reflecting pigment as well as the distal pigment has migrated into a position typical for a light-adapted eye. The proximal retinal pigment apparently undergoes no change in position.

5. Stalk extracts which were prepared from animals that were adapted to darkness, and which were injected into dark-adapted *Palæmonetes*, in the dark-room, proved to be only half as potent as extracts prepared from the eyes of light-adapted shrimps.

6. Extracts of eye-stalks from *Cancer irroratus, Libinia dubia, Uca pugilator*, and *Carcinides mœnas,* when injected into *Palæmonetes* which were adapted to darkness, also effected migrations of the distal and the reflecting retinal pigments. Stalk extracts from the eyes of *Callinectes sapidus,* in the concentrations used, had no effect on the retinal pigments of the test animals.

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BINARY ANTERIOR OCELLI IN ANTS

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The opinion that the anterior of the three ocelli of insects really represents a pair of intimately fused ocelli is generally accepted by morphologists and is briefly expressed in several recent entomological handbooks. Thus Snodgrass (1935, p. 533) remarks that "there is evidence that the median ocellus has been formed by the union of two primitive frontal ocelli," and Imms (1934, p. 77) states more explicitly that "the median ocellus exhibits evidence of a paired origin since the root of the nerve supplying it is double, whereas the nerve-roots of the other ocelli are single. In some insects (e.g. Odonata, *Bombus*) the median ocellus exhibits a bilateral structure which is never found in the remaining ocelli." Imms' mention of the Odonata seems to refer to Hesse's observations (1901, p. 384) on *Agrion,* the median ocellus of which "has the same structure as the lateral ocelli, except that it is symmetrical. Whereas in other insects the duplicity of this eye is merely suggested by the double optic nerves, a bipartite condition is shown in *Agrion* also by the pushing in from the rostral side for some distance of a wedge of indifferent cells between the retinulæ."

Leydig seems to have been the first to detect the double innervation of the anterior ocellus. In his paper on the arthropod eye published in 1864 he says: "Concerning the optic nerves it may be said that the nerve of the median ocellus has *two roots,* each of which arises in one of the halves of the brain," and refers to the illustration (Fig. 4) of the brain of the *Formica rufa* queen in his atlas of comparative anatomy (1864*a*). This beautiful figure was reproduced by Sharp in his well-known treatise on insects in the Cambridge Natural History (1895, Fig. 65, p. 119). Among the later anatomists who have confirmed Leydig's observations on the double nerve supply of the anterior ocellus, Janet (1905) and Berlese (1909) may be mentioned.

Additional indications of the binary origin of the median ocellus are its conspicuously greater size as compared with each of the posterior ocelli in most worker ants which possess these organs and its sole persistence after the posterior ocelli have disappeared. If, however, there could be any doubt of the paired origin of the organ this has been removed by the study of its development. As early as 1887 Patten, investigating the origin of the ocelli in *Vespa*, reached the following conclusions: "The *ocelli* of Vespa appear at the close of the larval period as *four* deep pits whose walls consist of a single layer of cells. The two anterior pits are situated close together, and soon fuse to form a single depression which develops into the anterior ocellus." . . . "The fact I desire to emphasize here, is that *the three ocelli arise from four single-layered pits, the median ocellus being formed from a coalescence of the two ventral ones*. The double nature of the median ocellus is also shown by the fact that, even in the latest stages, the root of the nerve is double, while that of the other two is single."

Apart from certain details such as the pit-like character of the ocellar primordia, Patten's observations have been confirmed by Redikorzew (1900), Zavřel (1902) and Caesar (1912). Although Redikorzew did not see the fusion of the two primordial thickenings in the honey bee, which he studied, he none the less observed the formation of the two nerves of the anterior ocellus and a very suggestive arrangement of the retinal pigment of all three ocelli. When first deposited in the retinulæ of the anterior ocellus the pigment has a remarkable bilateral distribution and that of each half is like the arrangement in each of the posterior ocelli. "From which it may be seen that each of the two halves of the median ocellus, so far as the pigment is concerned, has precisely the same structure as that of a whole lateral ocellus; in other words, it seems to be made up of two lateral ocelli."

Zavřel differs from Patten only in finding in *Vespa* that the four ocellar primordia are differentiated out of an even earlier, single "sense-plate." He gives some good illustrations of the double innervation of the anterior ocellus. Caesar has made the most thorough study to date of both the structure and development of the ocelli in ants. He describes the origin of these organs in the male of *Formica pratensis* as follows: "As in all the species of ants investigated the earliest primordia of the future ocelli make their appearance as a rule in the half-grown larvæ as four somewhat lens-shaped thickenings of the cerebral plate of the developing head-fold. Of these four primordia the two median, as shown most clearly in frontal sections, lie very close together and therefore constitute the double primordium of the future median ocellus, a proof that this ocellus has arisen phylogenetically by coalescence of two single ocelli." . . . "Concurrently with these developmental stages the lentigenous and retinogenous layers of the median ocellus fuse more and more intimately, but even in this stage a last indication of the former double origin of the whole primordium is retained in the two completely separate nerve-stands which connect it with the brain." The nearest analogue of the single anterior ocellus and its double nerve supply among animals other than insects is the unpaired nasal sac of the cyclostome fishes with its paired olfactory nerves.

I have been led to consult the literature above cited by finding in some 15 among more than 4,000 gynandromorphs (ergatandromorphs) of a large neotropical ant, *Cephalotes atratus quadridens,* that the anterior ocellus is replaced by two smaller contiguous ocelli (Fig. 1).[1] Obviously, in these specimens the original paired primordia of the retinæ, corneagen cells and corneæ have differentiated in the adult without coalescing. Although it seemed probable that similar doubling of the anterior ocellus might occur occasionally among otherwise normal insects, I could recall no cases among the many thousands of Formicidæ and other aculeate Hymenoptera which I have examined during the past forty years. Moreover, my colleagues, Professor C. T. Brues, Dr. J. Bequaert, and Mr. Nathan Banks, who have a very wide acquaintance with many orders of insects, were equally certain of never having seen an example of double anterior ocellus and, like myself, could recall no reference to such an occurrence in the entomological

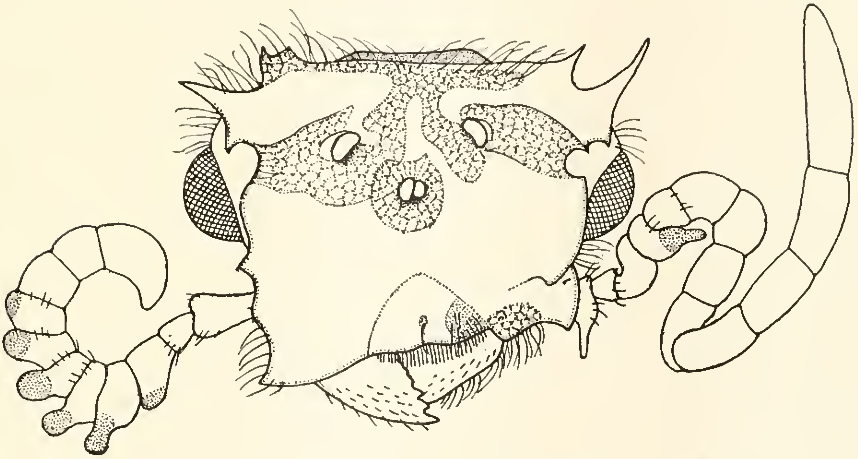

FIG. 1. Head of gynandromorph (ergatandromorph) of *Cephalotes atratus quadridens* Degeer, with binary anterior ocellus. One of several specimens. The large eyes, left mandible, thirteen-segmented left antenna, the stippled area and long hairs on the head and clypeus (except along its anterior border) and the papilla-like, stippled projections of the median joints of the right antenna are male characters.

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[1] This extraordinary number of gynandromorphs occurred in a single huge colony of *Cephalotes atratus quadridens* Degeer recently collected with great care by Dr. N. A. Weber in the island of Trinidad, B. W. I. A preliminary account of these anomalies was presented at the meeting of the British Association for the Advancement of Science in September, 1935. The final paper by Dr. Weber and myself has not yet been completed.

literature. At first sight, Comstock's general account of the ocelli in insects (1924) creates a suspicion that two ocelli may actually have been observed in some species. He says, p. 134: "When all [eyes] are present there are two compound eyes and, typically, two pairs of ocelli; but almost invariably the members of one pair of ocelli are united and form a single median ocellus. The median ocellus is wanting in many insects that possess the other two ocelli." On p. 135 he repeats this statement in a different form: "Of these [ocelli] there are typically two pairs; but usually when they are present there are only three of them, and in many cases only a single pair. When there are three ocelli, the double nature of the median ocellus is shown by the fact that the root of the nerve is double, while that of each of the other two is single." Since Comstock cites no cases of binary anterior ocellus, one suspects that the word "typically" in both these quotations is probably employed in the sense of "originally" or "phylogenetically."

After considerable search, however, I find that double anterior ocelli do occur in at least two species of myrmicine ants and I therefore believe that further investigation may reveal their presence in some other insects. The ant which exhibits this peculiarity most frequently is the huge-headed soldier, or worker maxima of the large neotropical leaf-cutting and fungus-growing *Atta cephalotes*. Linnaeus in his original description of this insect makes no mention of the ocelli, and Degeer (1773), who described it more carefully, expressly states that "the three smooth eyes are lacking." F. Smith in 1858 seems to have been the first to call attention to the large median ocellus in the worker maxima of *cephalotes* as distinguishing it from the same caste in *Atta sexdens*. According to Forel (1904) the maximæ of the former species "have three ocelli, of which the anterior is large, the two posterior small."

I have examined 300 maxima workers from series taken from some 25 colonies. Of these specimens, which comprise the typical *cephalotes* from the Guianas, Venezuela, and Trinidad, the subspecies *integrior* Forel (Brazil), the subspecies *opaca* Forel (Central America and northwestern South America) and *gorgo* subsp. nov. (Panama), more than 60 per cent have the anterior ocellus more or less distinctly divided.[2] In some of the series the percentage is even higher than 60, in others

FIG. 2. Front and ocelli of twelve specimens of the worker maxima of *Atta cephalotes*. *a*, subsp. *opaca* (British Honduras); *b* and *c*, subsp. *gorgo* subsp. nov. (Panama); *d*, *cephalotes*, typical (British Guiana); *e* and *f*, subsp. *gorgo* (Panama); *g*, *cephalotes*, typical (British Guiana); *h*, subsp. *opaca* (Ecuador); *i*, *cephalotes*, typical (Trinidad); *j*, subsp. *opaca* (Ecuador); *k*, subsp. *opaca* (Costa Rica); *l*, subsp. *opaca* (Ecuador).

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[2] For a brief revision of these and other forms of *Atta* see my paper "Taxonomic Notes on the Ants of the genus Atta" (in preparation).

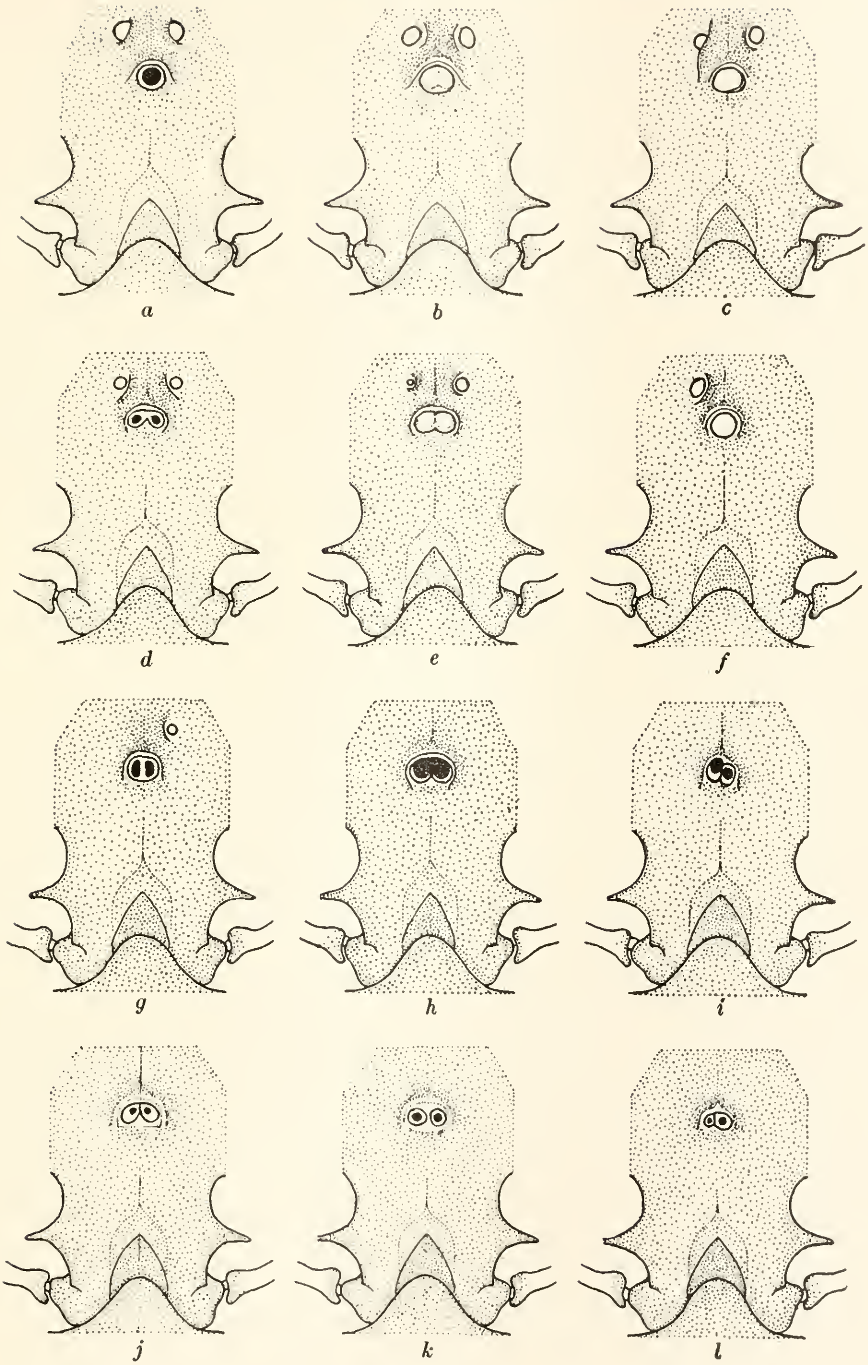
a
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Fig. 2.

less. Thus in a series of 73 maximæ from a single flourishing and very pugnacious colony which Dr. William Beebe and I excavated at Kartabo, British Guiana, September 1, 1920, 93 per cent have a distinctly bipartite anterior ocellus. As shown in the 12 illustrations (Fig. 2), the ocelli are highly variable. Some specimens (*a–e*) possess all three ocelli but one of the posterior is frequently smaller than the other (*c*, *e*) or as in *f* and *g* one of the pair has disappeared. In all cases each of posterior ocelli is decidedly smaller than the unpaired anterior ocellus. In alcoholic specimens this may have a single pigmented retina more or less clearly visible through the clear, undivided cornea (*a*), but all

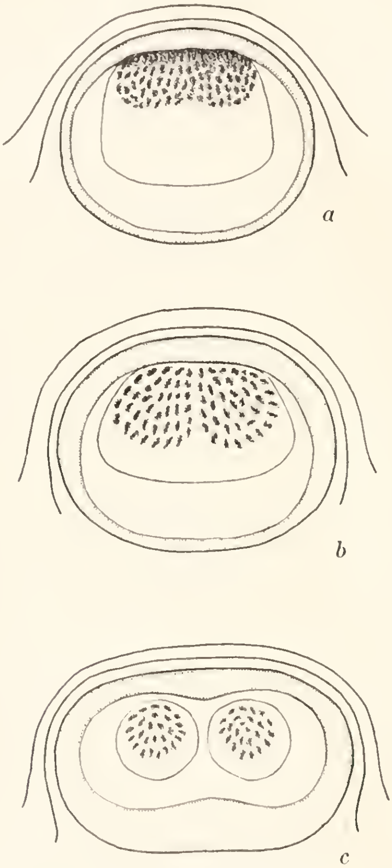

FIG. 3. Anterior ocelli of three maxima workers of *Atta cephalotes* from British Guiana seen from inside of head and showing distribution of retinal pigment under high magnification. *a*, retina with only slight and *b*, greater tendency to bipartition of retina. *c*, with two widely separated retinæ.

stages may be observed from partially to completely divided corneas and/or retinæ (*b, d, e, g, i–l*). Occasionally one of the two members may be smaller (*l*) than the other or placed at a lower level on the front (*i*).

Portions of the front of maxima workers of *cephalotes* stained and mounted *in toto* and viewed from the inner surface of the integument under a high magnification showed little that could not be inferred from the outer surface, except the more definite arrangement of the pigment in the ommatidia. In the three preparations represented in Fig. 3 the amount of pigment in the anterior ocellus is approximately equal to that of both posterior ocelli, when these are present. In all my preparations the pigment of the anterior ocellus shows also that there are two retinæ which in some specimens are contiguous or even fused, in others separated by a very appreciable gap. The material, however, is so old and poorly preserved that other histological details cannot be discerned. The same statement applies to my sections of heads of pupal maxima workers. Hence before further investigation can be undertaken freshly fixed material will have to be obtained.

The other ant in which I have seen a double anterior ocellus is the Indomalayan *Pheidologeton diversus* Jerdon, a species which possesses a huge worker maxima like the same caste in *Atta cephalotes*. Usually only the large anterior ocellus is present, but occasional specimens also retain minute posterior ocelli. Unfortunately, my series of maximæ of this ant is rather small, but in one specimen belonging to the variety *laotina* Santschi from Saigon, the cornea of the anterior ocellus is distinctly bipartite.

The anterior ocellus in the largest workers of *Atta cephalotes* and *Pheidologeton diversus,* though large compared with either of the posterior ocelli, when they are present, is, nevertheless, a vestigial structure compared with its homologue in the cospecific male and female. When bipartite it obviously exhibits a retardation of development and persistence in a stage corresponding to the original paired hypodermal thickenings of the larva before or just after their union in the middorsal line, though the corneagen and retinogen cells are differentiated beyond this stage, as shown by the secretion of the cornea and the deposition of retinal pigment. I fail, however, to detect any differentiation of rhabdomes, but this is probably due to the poor preservation of the material.

Since, as a very general rule, the two primordia of the anterior ocellus in adult insects fuse to form a single organ, we are naturally led to inquire whether in certain species there may not be a coalescence of the primordia of both pairs of ocelli. There is, indeed, some evidence of such an occurrence. It has long been known that what authors have interpreted as the posterior pair of ocelli in Plecoptera (Ephemerida) differ from those of all other insects in having an undeveloped cornea and beneath it a cellular lens. Now Seiler (1905) has shown that the anterior ocellus of *Ephemera vulgata* is really tripartite and is supplied by *three* nerves. This investigator is therefore inclined to believe that the so-called posterior ocelli of ephemerids are not homologous with the posterior ocelli of other insects and that the anterior ocellus is really a fusion of all three typical insect ocelli. The median of the three ocellar nerves, as shown in his figure, is twice as thick as either of the others and may therefore have arisen by coalescence of the pair belonging to the anterior ocellus. At any rate, renewed comparative study of the ocelli of ephemerids might yield results of considerable morphological interest, because Seiler's work clearly suggests that the ancestors of these insects were provided with three instead of two pairs of simple, in addition to the single pair of facetted eyes.

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THE BIOLOGY OF PSEUDOCALANUS MINUTUS IN THE GULF OF MAINE AND BAY OF FUNDY

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Introduction

The present paper forms the second of a series on the biology of zoöplankton species in the Gulf of Maine and Bay of Fundy, with special reference to production and dispersal. As in the case of *Calanus finmarchicus* (Fish, 1936) there have been two objectives; first, a determination of breeding seasons, annual number of broods, and rate of growth; second, an evaluation of different spawning areas and dispersal of eggs and larvæ. For a description of the area covered, location of stations and methods, the reader is referred to the aforementioned report. The studies were carried on between July 28, 1931 and September 29, 1932 for the International Passamaquoddy Fisheries Commission.

Regional Distribution of the Adult Stock[2]

Pseudocalanus minutus was found by Bigelow (1926, pp. 276–277) to be nearly as universal as *Calanus* in the Gulf of Maine, distributed indifferently in the coastal zone, the deeper parts of the basin, and on offshore banks. It was somewhat more nearly universal close along shore but the numbers actually present averaged larger in the basin, entrant channels, and along the offshore slope. In 1931 and 1932 it was found generally distributed in both neritic and offshore waters.

Being more indifferent to neritic environmental factors than *Calanus* and with an apparently somewhat greater thermal range, *Pseudocalanus* occurs to some extent throughout the year in inshore waters north of Cape Cod. However, like other boreal arctic species, its numbers decline rapidly with rising summer temperatures in shallow areas, and offshore Bigelow (Ibid., p. 277) observed that there is a general tendency to leave the uppermost stratum in April and May.

In Passamaquoddy Bay in 1932 there was a decline in the relative percentage of *Pseudocalanus* from 84.7 per cent on June 20 to 2.8 per cent on July 30, with a corresponding reduction in the volume of plankton from 26.7 cc. to 1.9 cc. Later it rose again to 12.1 per cent accompanied by an increase in volume to 13.3 cc. on September 23. McMurrich's unpublished data from St. Andrews in 1916 accord with these results.

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[1] Contribution No. 94.

[2] "Adult stock" includes late copepodites (for the most part stage V) appearing in the coarse net hauls.

In Frenchmans Bay, *Pseudocalanus* remains throughout the summer as one of the most important members of the zoöplankton community in the cold bottom water, but its center of abundance was found by day at a slightly higher and warmer level than that of *Calanus*.

South of Cape Cod *Pseudocalanus* largely disappears from the neritic zone during the summer months. In the Woods Hole region it remains abundant until May, declines rapidly in June after the water mass rises above 15° C., and disappears at about 20° C., reappearing usually in October (Fish, 1925, Fig. 45). *Calanus* occurs only during the coldest months in this area and is rarely ever numerous. Farther south in Chesapeake Bay *Pseudocalanus* is regularly found in the inner bay in the spring, but *Calanus* appears to be confined to the outer bay, where it is taken only in winter (Wilson, 1932, p. 13).

It would appear, therefore, that *Pseudocalanus* regularly outnumbers *Calanus* in observed neritic localities south of Cape Cod, and north of the Cape a similar condition occurs occasionally in some coastal areas during the early summer.

PRODUCTION AND DISPERSAL OF EGGS AND LARVÆ

Spawning Areas

The distribution of eggs and early nauplii in 1931 and 1932 indicates that *P. minutus* spawns throughout the Gulf and Bay of Fundy. Even in neritic areas such as Passamaquoddy Bay where no evidence of *Calanus* propagation was found, 2,847 *Pseudocalanus* eggs per minute were taken on June 20, and 760 per cubic meter at the end of July in 1932.[3]

There is, however, considerable variation in the extent of propagation in different parts of the region. If the *Pseudocalanus* population in the inner Gulf and Bay originated largely in the western coastal area, as appears to be the case with *Calanus*, one would expect the quantitative distribution of larvæ of the two species to be very similar. The data obtained in 1932 indicate striking differences. Whereas the numbers of *C. finmarchicus* in all stages declined uniformly to the eastward (Fish, 1936, Fig. 11) and quantitative fluctuations in the western area were reflected throughout the region, in *P. minutus* the number of larvæ in the eastern basin and particularly in the outer part of the Bay of Fundy was usually as large or even larger than west of Mt. Desert (Fig. 1).

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[3] Just outside of the bay in outer Quoddy waters (Sta. 5) *Calanus* eggs totalled 7,608 per minute and *Pseudocalanus* 37,018 on June 20, and *Calanus* 710 per cubic meter and *Pseudocalanus* 800 on July 30.

This condition could have resulted either from intensive local spawning or large contributions from other areas. In regard to the first possibility, evidence of immigration in the horizontal distribution of all advanced larval stages of *P. minutus* indicates that production of this species in the eastern coastal region is on the whole probably no more successful than that of *C. finmarchicus* (Ibid., p. 140), *Sagitta elegans* (Huntsman and Reid, 1921, p. 110), and fishes having pelagic eggs (Bigelow, 1926, pp. 70–73; Huntsman, 1918, p. 65, 1922). Considering other possible sources of supply, as propagation in the western coastal area does not seem to have been sufficient to account for the increasingly large numbers of *Pseudocalanus* occurring farther east (up to 215,698 late nauplii per minute in the Bay of Fundy in June) as the season advanced (Fig. 7*B*), it is probable that the principle spawning area of *P. minutus* is located in the outer Gulf where Bigelow (1926, p. 277) regularly found the species most abundant. The western coastal area appeared to be an important source for the first brood (Fig. 5*A*) in 1932, but thereafter declined until in mid-August there was little evidence of appreciable propagation except in a restricted locality represented by the innermost stations between Casco and Penobscot Bays (Figs. 8*A* and *B*).

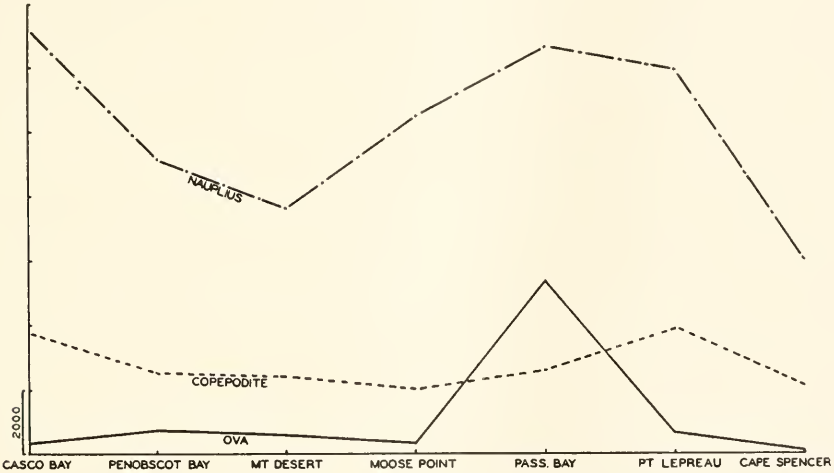

FIG. 1. Mean numbers of ova, nauplius and copepodite stages of *P. minutus* per minute of towing for the period April–July, 1932.

Breeding Seasons

The breeding periods of *P. minutus* appear to be somewhat similar to those of *C. finmarchicus*. As in the latter species, propagation begins earlier in the southern portion of its range and there appear to be a greater number of annual broods.

South of Cape Cod *Pseudocalanus* enters neritic waters in late October and breeding must soon follow because in 1922–23 early copepodite stages appeared at Woods Hole by the middle of December (Fish, 1925, p. 144). A maximum of copepodites in January was followed by a rise of adults in February. A second brood of less importance appeared in March and April. Some no doubt enter the region from the north where vernal augmentation takes place at this time, but in view of the abundance of adults at Woods Hole in February, it seems probable that the greater part of the March–April copepodites were of local origin. With the rise in temperature in May the numbers declined, and the species practically disappeared from inshore waters in June. Subsequent broods no doubt occur offshore where *Pseudocalanus* is found throughout the summer. The southern limit of successful propagation is not known but its presence regularly in Chesapeake Bay in winter and spring (Wilson, 1932) would suggest that there may be some successful spawning at that latitude during the cold months.

North of Cape Cod, Willey (1918, p. 187) reported females with eggs and attached spermatophores in August, and Bigelow (1926, p. 282) concluded that " the seasonal fluctuations in the numerical strength of the stock point to breeding as taking place most actively from June until September and to the entire gulf as its site."

Ruud's records (1929, pp. 67–68) indicate that the cycle in Norwegian waters is somewhat similar to that of *C. finmarchicus*. Spawning begins in March off Möre and probably earlier off the west coast of Norway. In 1926 there was a maximum of copepodite stages I–III on April 28–29. All copepodite stages had another maximum on July 20. This would suggest production in March, the young reaching maturity in May–June and the second generation attaining copepodite stages by July 20. Several observers have reported the presence of copepodite stages and mature adults in northern waters during the summer (Störmer, 1929, p. 31; With, 1915, p. 61; Bogorov, 1932).

In the Gulf of Maine and Bay of Fundy the seasonal distribution of young stages in 1931 and 1932 indicates that propagation begins in March and continues in a succession of generations throughout the region until September.

Regional variation.—Eggs of *P. minutus* appear at progressively later periods in the outer Gulf, western area, and Bay of Fundy, and, on the basis of this regional variation in the time of spawning, it is possible to distinguish three major breeding stocks.

These stocks are not permanently restricted to their respective localities, but merely represent delayed maturation of that portion of the winter population of *P. minutus* which happens to be located in the eastern part of the Gulf and Bay of Fundy during the spring, and earlier maturation in areas to the westward where the water mass responds more rapidly to vernal warming.[4] This is evident from the fact that the progeny of all stocks disperse and appear to become established over much if not all of the region, and also because within a region with a dominant drift it would be impossible to have permanently localized zoöplankton stocks without bottom stages. From the surface to the bottom the plankton population in any part of the open Gulf and Bay is in varying degrees a transient one. However, since the annual cycle in the three major areas starts at different times, with approximately the same interval of development (p. 200), one would expect the distinct breeding periods of the three stocks to be continued, as in *Calanus* (Fish, 1936, p. 130), in subsequent generations no matter where they might be dispersed. This is indicated in the present material.

In the following pages symbols will be used to designate the different stocks originating in the three general regions in the spring, and their respective progeny wherever distributed during the remainder of the season: *A* indicating the outer gulf stock, *B* that of the western coastal area, and *C*, that originating east of Mt. Desert in the inner Gulf and centered in the Bay of Fundy.

Annual Cycle in Different Breeding Stocks

Tracing the succession of broods in the three breeding stocks of *P. minutus* is more complicated than in *C. finmarchicus*. Unlike the latter species, where the bulk of the population of the Gulf apparently originates in one area (western) and can easily be traced, the broods of *P. minutus* are everywhere so largely depleted before copepodite stages are reached, that the particular stock which happens to be in early larval stages at the time dominates throughout the Gulf and Bay. Copepodite stages were almost always relatively sparse and in the present collections rarely formed appreciable maxima.

Since the succession of generations is most clearly defined in the stock maturing in the western coastal region in the spring, this stock (*B*) will be considered first. There appear to be at least three and possibly four breeding periods: March–April, May–June, July–August, and September. As seen in the quantitative distribution in successive

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[4] It is probable that the transfer of adults from one area to another is relatively much slower than that of larvæ because, by descending during the daylight hours, they pass beyond the influence of the surface into a slower dominant drift.

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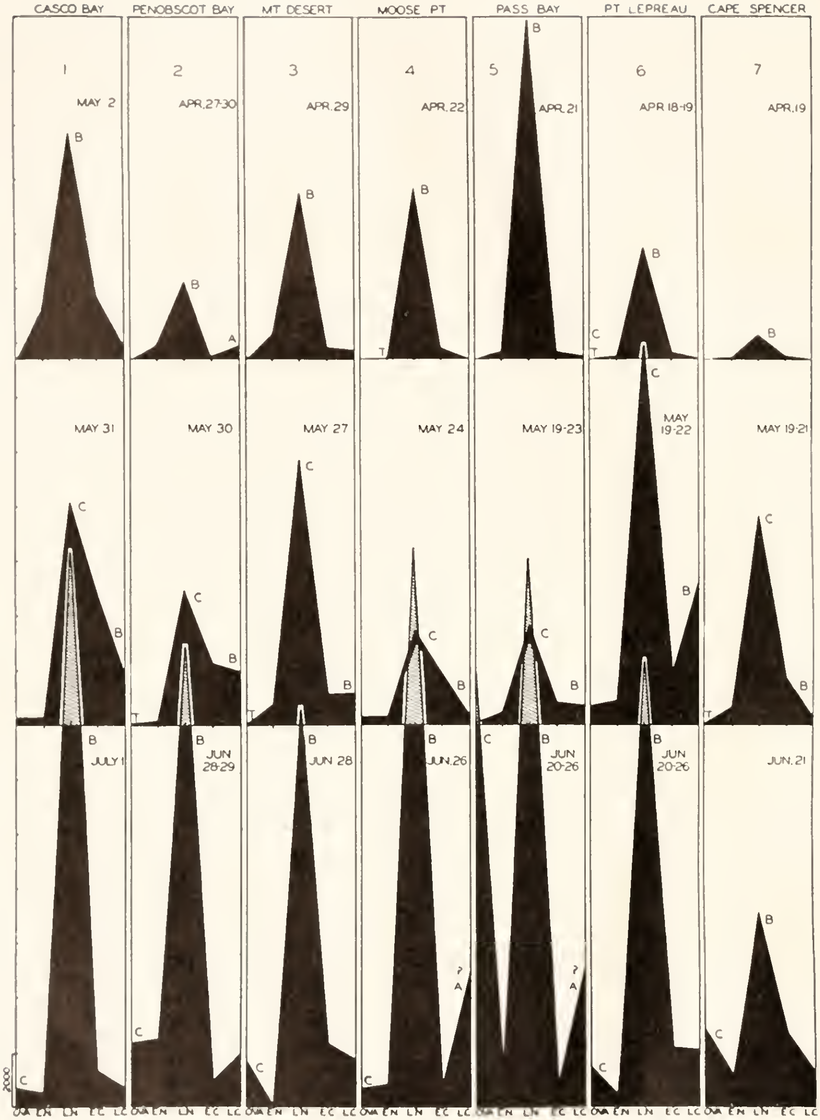

Fig. 2. Changes in the composition of the population of *Pseudocalanus minutus* (developmental stages) in seven sections between Casco Bay and Cape Spencer from April to June, 1932. Number per minute of towing. Stages recorded as: ova, early nauplii (I–III), late nauplii (IV–VI), early copepodites (I–III), and late copepodites (IV–V). *B* indicates stock originating in the western area in the spring, *C* indicates that of the eastern Gulf and Bay of Fundy, and *A*, the outer Gulf stock. *T*—trace.

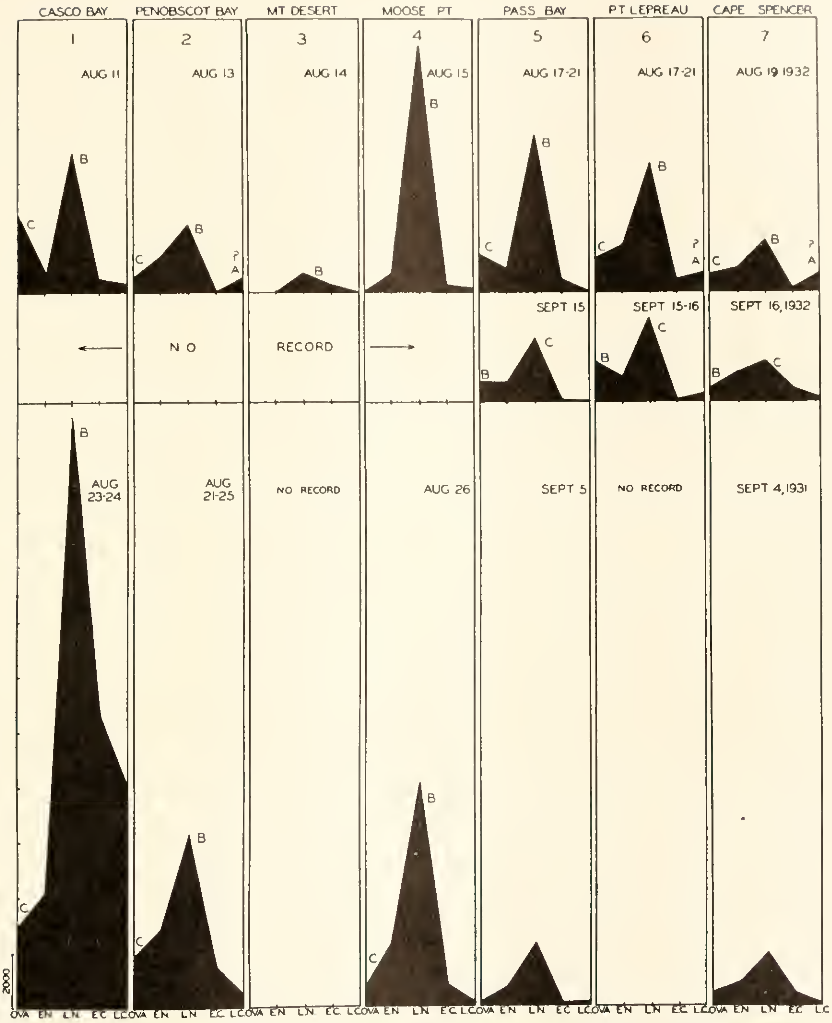

FIG. 3. Composition of the population of *Pseudocalanus minutus* (developmental stages) in August and September, 1931 and 1932. Number per cubic meter. Stages and symbols as in Fig. 2.

months, the first brood in advanced nauplius stages in late April had reached late copepodite stages on May 19–22 (Fig. 2, Section 6), and mainly completed the final moult by May 31 (Section 1). What is assumed to have been the second and largest western brood was represented by a maximum of late nauplius stages in the latter part of June. It would thus appear that the cycle during the early season is completed in about 60 days. The third brood, hatching on July 30 (Fig. 4), probably developed somewhat more rapidly with warmer summer temperatures and attained late nauplius stages by mid-August in the western area (Fig. 3). Even in the eastern Gulf at lower temperatures a large part of this stock passed from early to late nauplius stages between August 8 and 15, as indicated in Table 1. Eggs entering the Bay of Fundy on September 15–16 would suggest a fourth generation but further data from the Gulf at this season are needed. It seems probable that a considerable percentage of the third crop remains in late copepodite stages to form the winter stock.

TABLE I

Stage	August 8, Sta. 15	August 15, Sta. 33
Egg	1,132	
Early nauplius	4,335	565
Late nauplius	5,842	12,060
Early copepodite	1,695	565
Late copepodite	377	377

In stock C, originating in the eastern area, there is evidence of three breeding periods: April–May, June–July, and August. Local propagation was just beginning in the Bay of Fundy on April 19 (Fig. 2, Section 6) and a month later nauplius stages dominated. With a cycle of 60 days, eggs on June 20 (Fig. 4) would represent a second brood which by August 11–21 had matured and were spawning (Figs. 3 and 8*A–B*). A third brood is indicated by a maximum of late nauplius stages in the Bay of Fundy on September 15 in 1931 (Fig. 4), and September 15–16 in 1932 (Fig. 3).

Some less definite indications of a possible succession of generations in stock *A* after April are to be found in the data from the coastal region and the Bay.[5] In 1932, on June 20–26, when stock *B* was mainly in late nauplius stages, and the second brood of eggs of stock *C* had just appeared, a smaller maximum of late copepodite stages (Fig. 2, Sections 4 and 5) in the eastern basin may have represented a second brood of immigrants from offshore (Fig. 9*A*). With a developmental period of two months this would be expected. There is also an indication of a third crop of late copepodites on August 11–21, particularly in the western area (Fig. 3, Section 2) and the Bay of Fundy (Sections 6 and 7), but the numbers are too small to be of any real significance (Fig. 9*B*).

Although there was no detectable trace of stock *A* in May (Fig. 2), on the basis of the developmental period, the succession of subsequent maxima just described would appear reasonably indicative were it not for another complicating factor—the relatively small numbers of progeny attributable to this stock. There appears to be no logical reason for the depletion of one breeding stock of *Pseudocalanus* and the survival of another in the same locality. It is probable, therefore, that following the first generation of stock *A*, due to overlapping spawning periods, subsequent broods of this stock and stock *C* cannot with certainty be distinguished. At least a part of stock *A*, in copepodite stages in the outer Gulf on April 11–12 (Fig. 5*B*), would be expected to have matured and started to spawn by the time eggs, forming the first brood of stock *C*, appeared in the Fundy region in the latter part of the month. In this case subsequent maxima, appearing in each instance one month after maxima of similar stages of stock *B*, would represent the combined stocks *A* and *C*, and the maxima of late copepodite stages[6] in late

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[5] In *Calanus*, either spawning in the outer gulf and western area coincides or the number of individuals of the offshore stock is insufficient to exert a detectable effect on the larval population of the inner gulf and bay.

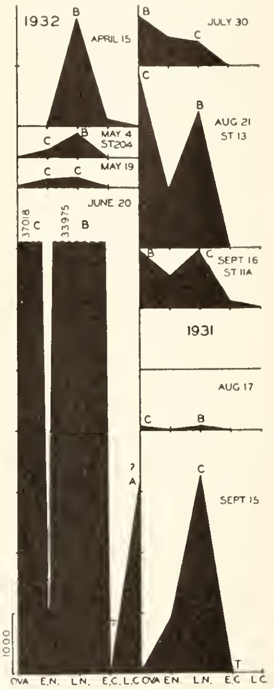

FIG. 4. Composition of the population of *P. minutus* in developmental stages at Station 5 (unless otherwise noted) in the New Brunswick area of the Bay of Fundy, showing the succession of generations of the (*B*) western, (*C*) eastern, and (*A*) outer Gulf stocks. *T* indicates trace. April–June: number per minute. July–September: number per cubic meter.

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[6] These copepodites, according to this interpretation, were probably derived from eggs appearing near the end of the previous spawning period and might be indicative of either one or both stocks.

June and August should be grouped with eggs appearing at the time. Further evidence in support of this contention will be presented later (pp. 209–211). In any event, with a similar rate of growth, it is reasonable to conclude that in stock *A* there are four broods, each of which appears about a month earlier than in stock *B* and two months or approximately one generation in advance of stock *C*.

Annual variation.—Observations at corresponding stations were made approximately two weeks later in 1931 than in 1932. As in the case of *Calanus* (Fish, 1936, p. 130) the season appeared about equally advanced in the western area, the slight increase in the percentage of copepodite stages off Casco Bay in 1931 (12 days later) being expected (Fig. 3).

In the central area east of Mt. Desert, and the Bay of Fundy, the fact that the relative numbers in comparable stages were approximately the same in the two years, would indicate that in 1931 the season was delayed about two weeks, a condition also according with *Calanus* (Ibid., p. 130).

TABLE II

Stage	Central Area		Bay of Fundy	
	1931 (Aug. 26)	1932 (Aug. 8–15)	1931 (Sept. 1–5)	1932 (Aug. 17–21)
Egg	750	566	225	1,041
Early nauplius	2,204	1,414	800	1,091
Late nauplius	8,117	8,810	2,937	4,112
Early copepodite	799	989	275	302
Late copepodite	188	330	112	442

Mortality in Developing Larvæ

The degree of mortality in developing larvæ of *P. minutus* can best be seen by comparing the relative abundance of different stages with those of *C. finmarchicus.* Of the two species *Pseudocalanus* must be by far the more prolific. The large number of spermatophores frequently found attached to individual females (Willey, 1919, p. 187, records as many as 24) suggests high egg production. The ovisac when attached contains usually from one to three eggs, but this may prove to be an adaptation for temporarily retaining the eggs to insure fertilization. Sars (1903, p. 21) observed that the sac is so fragile that it easily becomes detached at the slightest touch, and in examining Bigelow's collections (1926, p. 281) taken throughout the period of propagation in the Gulf of Maine, Wilson failed to find a single egg-bearing female. Individuals with from one to two attached eggs were frequently taken in 1932, but to account for the numbers of free eggs of this species present at times in the region, several hundred must be produced by each individual. During the season of 1932, as shown in Table III, although adults (including stage V) were outnumbered by *Calanus* in the total region by an average ratio of over 4 to 1, eggs and larvæ of *Pseudocalanus* dominated at all times with an average ratio of 2.5 to 1.

TABLE III

1932	Eggs and young (all stages)		Relative percentage of adults	
Month	P. minutus	C. finmarchicus	P. minutus	C. finmarchicus
April	7,126	2,011	12.4	25.7
May	11,610	7,454	12.7	56.4
June	24,967	10,588	16.8	44.7
August	6,435	3,135	2.1	35.6
September	4,378	794	1.3	37.3

April–June: number per minute. August–September: number per cubic meter.
Relative percentage of adults (including stage V) in the zoöplankton population.

TABLE IV

Section	1932	P. minutus	C. finmarchicus
Casco Bay	April–June	17,248	13,137
	August	8,049	4,466
Penobscot Bay	April–June	12,442	9,490
	August	4,897	4,342
Mt. Desert	April–June	13,635	7,160
	August	956	1,860
Moose Peak	April–June	12,834	3,679
	August	10,181	5,184
Passamaquoddy Bay	April–June	20,494	4,749
	August–September	6,072	2,145
Pt. Lipreau	April–June	16,424	6,873
	August–September	7,164	1,516
Cape Spencer	April–June	8,893	1,665
	August–September	3,813	578

April–June: number per minute. August–September: number per cubic meter.

This is seen in Table IV to occur everywhere in the inner Gulf and Bay, the differences being greatest east of Mt. Desert.

Unlike *Calanus,* in which the depletion appears to be most extreme following the final moult (Nicholls, 1933, p. 95; Fish, 1936, p. 136), the decline in *Pseudocalanus* seems to be greatest in late nauplius stages.

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There must also be a second critical period during maturation, which affects a far greater percentage of the stock than in *Calanus*, to establish the adult ratio because copepodite stages of *Pseudocalanus* exceeded those of Calanus during both the early and late summer seasons. (See Table V.)

Physical environmental factors appear to offer no logical cause for the depletion throughout the Gulf and Bay, and in the case of the late nauplii it seems to be attributable to destruction by enemies. Were there a lack of food, the relative decline in *Calanus* and *Pseudocalanus* larvæ would be expected to be similar.

Dispersal and Result on Regional Abundance of the Stock

The dispersal of eggs and larvæ from production centers and subsequent distribution of the adult stock are largely dependent on non-tidal circulation. The surface drift in the Gulf of Maine is counter-clockwise, eddying around a central vortex. Eggs and larvæ produced in the western area would tend to be transported offshore and eastward around the outer margin of the Gulf. Outflow around the eastern end of Georges Bank as well as past Cape Cod would carry some of the larvæ, particularly those near the periphery of the drift, out of the region. However, an appreciable portion of the migrant stock or its progeny would be expected to remain within the Gulf completing the circuit across the entrance of the Bay of Fundy and westward along the coast of Maine, and some would continue eastward into the Bay along the Nova Scotian side.

TABLE V

Total region 1932	April–June (Mean number per minute)		August–September (Mean number per cubic meter)	
Stage	P. minutus	C. finmarchicus	P. minutus	C. finmarchicus
Nauplius	10,568	2,160	4,476	638
Early copepodite	1,468	1,103	230	48
Late copepodite	1,079	514	292	10

One would therefore expect to find larvæ from the western area circling the outer Gulf and, with contributions from the latter area, supplementing the stock in the eastern basin and Bay of Fundy. Similarly, the greater number of eggs and larvæ originating in or entering the Fundy region would, if they survived, leave the Bay in the outflow which passes east and south of Grand Manan and tend to be dispersed westward in the inner gulf.

Dispersal in 1932.—Dispersal of individual stocks through successive generations has been traced by the monthly changes in the distribution of larvæ. Although stages ranging from ova to adults could usually be found almost everywhere in the region at any time, the maximum numbers, particularly of stock *B*, were in stages sufficiently separated from those of the other stocks to permit detection (Figs. 2 and 3).

Stock B.—Late nauplius stages, representing the first brood of that portion of the *P. minutus* population located in the western Gulf in the spring, were widely distributed throughout the region with local concentrations in the western and eastern basins by late April (Fig. 5*A*). A considerable part of the parent stock must have been transported well around the outer Gulf before spawning to account for such large numbers of nauplii in the Fundy region. There is a possibility, of course, that the larvæ might have been locally produced, but the sparseness of earlier stages east of Mt. Desert in the inner Gulf (Figs. 2 and 3) at this time and the appearance later of a distinct eastern brood (p. 200) warrants the conclusion that they were derived from parents transported from the western Gulf.

The concentration of late nauplii in a limited area off Halifax (Fig. 5*A*) indicates that the season, as in the case of *Calanus* (Fish, 1936), was as far advanced there as in the western coastal region. In the intervening region late nauplii were not found numerous until May, a condition somewhat comparable with the Bay of Fundy.

From April to May stock *B* declined greatly in numbers and by the time late copepodite stages were reached at the end of the latter month, quantities in excess of 1,000 per minute were found only in the inner Gulf west of Mt. Desert and over the central basin of the Bay of Fundy (Fig. 6*C*). The latter were no doubt migrants from the western or outer Gulf because copepodites were still very sparse as far west as Mt. Desert in the path of the outflow from the Bay, where one would have expected to find the stock present in the Fundy region in April if it had survived.

By the end of June important changes in the picture were noticeable. The second brood of stock *B* in late nauplius stages was, like the first brood in May, centered in the western coastal area and Bay of Fundy. In view of evidence of unsuccessful production in the latter area (p. 195), and the large number (21,410) of late nauplii in the entering drift on the Nova Scotian side, it seems probable that the April brood of stock *B* had become established in the general region of Georges Bank and the second brood of migrant larvæ from the western area were now being supplemented by increasingly large contributions from the outer Gulf. It was at this time (June 21) that the immense swarm mentioned on page 195 was encountered off St. John (Fig. 7*B*).

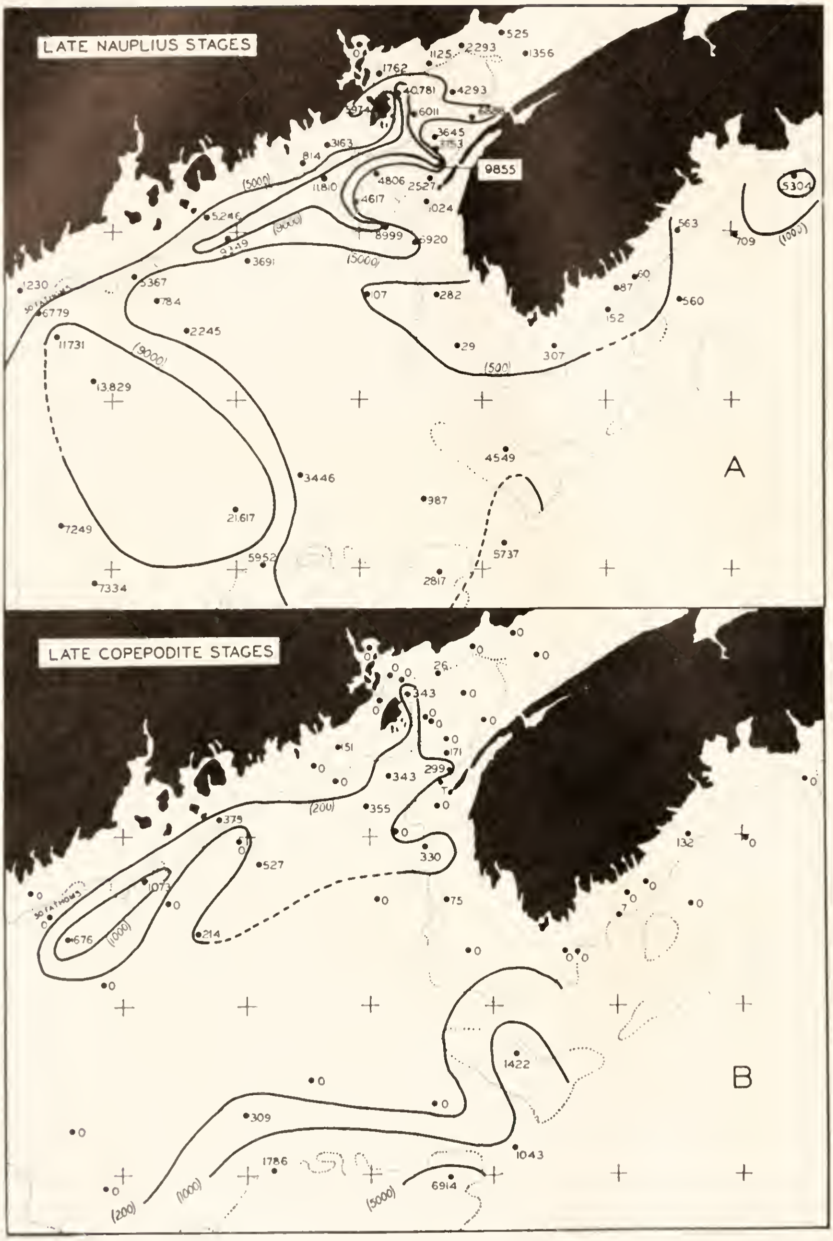
LATE NAUPLIUS STAGES
525
2293
1356
1125
1762
40,781
4293
6011
3645
3163
814
9855
11810
4806
2527
4617
1024
5304
5246
8999
6920
563
709
3691
1230
5367
784
107
282
60
87
560
6779
152
2245
11731
29
307
13,829
4549
3446
A
387
21,617
7249
5737
5952
2817
7334
(5000)
(9000)
(500)
(1000)
30 FATHOMS
LATE COPEPODITE STAGES
26
343
171
299
151
343
355
379
527
330
132
75
1073
676
214
1422
B
309
1043
1786
6914
(200)
(1000)
(5000)

Fig. 5. Occurrence of late nauplius stages (A) of the western stock, and late copepodite stages (B) considered indicative of offshore propagation, in April, 1932. Number per minute of towing.

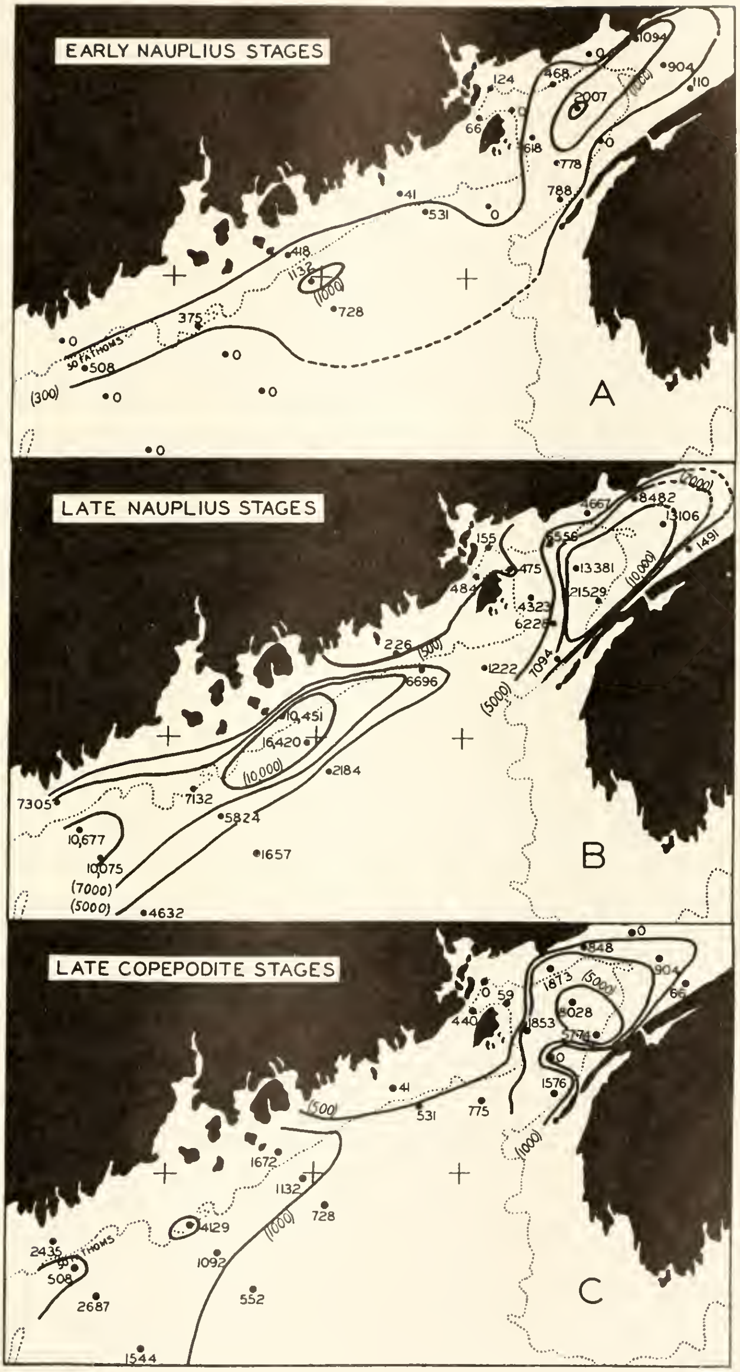
EARLY NAUPLIUS STAGES
1094
904
110
468
124
2007
(1000)
66
618
778
788
41
531
418
1132
(1000)
728
375
50 FATHOMS
508
(300)
A
LATE NAUPLIUS STAGES
(2000)
8482
4667
13106
155
5556
1491
475
13381
484
4323
21529
(10,000)
6226
226
(500)
1222
1094
6696
(5000)
10,451
16420
(10,000)
2184
7305
7132
5824
10,677
10,075
1657
(7000)
(5000)
4632
B
LATE COPEPODITE STAGES
848
904
1873
(5000)
66
59
440
3028
1853
5174
1576
41
(500)
531
775
(1000)
1672
1132
728
4129
(1000)
2435
50 FATHOMS
1092
508
552
2687
1544
C

Fig. 6. Occurrence of nauplius stages (*A–B*) of the eastern stock, and late copepodite stages (*C*) of the western stock in May, 1932. Number per minute of towing.

There can be no doubt that by mid-August the larger part of the *P. minutus* population was originating in offshore waters. The third brood of stock *B*, by this time in late nauplius stages (Figs. 3 and 4), was, in the inner Gulf, definitely centered in the eastern basin and along the Nova Scotian side of the Bay of Fundy (Fig. 8*C*). Immigration of this population from the outer Gulf is indicated by the continued scarcity of larvæ in the New Brunswick area and the coastal zone of eastern Maine beyond the immediate influence of the drift. The stock in the western area had declined until only at the innermost station off Casco Bay were more than 5,000 late nauplii per cubic meter taken.[7] If these conditions can be considered normal, then once the first brood of the stock originating in the western area in April becomes dispersed throughout the region, a considerable portion of the second brood, and

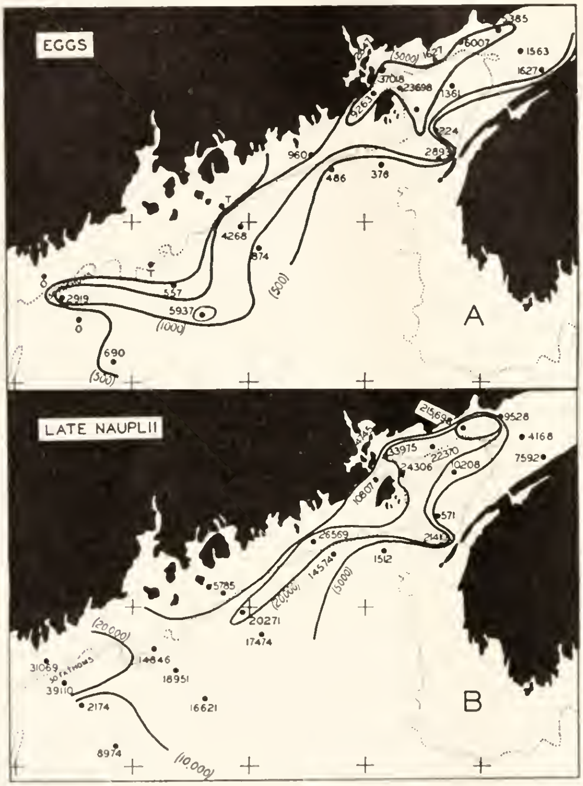
EGGS
385
6007
1621
(3000)
1563
1627
37018
23698
1361
9263
224
2893
960
486
378
T
4268
874
T
0
557
2919
5937
(1000)
(500)
0
690
(500)
A
LATE NAUPLII
215698
9528
4168
7592
4145
33975
22370
24306
10208
10807
571
2141
26569
14574
1512
5785
(20,000)
(5000)
20271
17474
(20,000)
31069
50 FATHOMS
14846
18951
39110
2174
16621
8974
(10,000)
B

FIG. 7. Occurrence of eggs (*A*) of the second eastern brood and late nauplii (*B*) of the second western brood in June, 1932. Number per minute of towing.

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[7] Possibly the large population of predatory plankton animals in this region in summer depletes the *P. minutus* larval stock.

the greater part of the third, are derived from individuals establishing themselves in the general region of Georges Bank.

Autumn dispersal in the Gulf could not be traced as observations were restricted to the Bay of Fundy after August. By September both eggs (presumably the fourth brood of stock *B*) and larvæ of *P. minutus* had declined greatly in the Bay.

Stock C.—Originating from that portion of the *P. minutus* winter population maturing in the eastern Gulf and Bay of Fundy, stock *C* is believed to have been represented by small numbers of eggs at Station 37 in the Bay on April 20. Vernal augmentation must have just begun because eggs were not found elsewhere in April.

Differing from stock *B*, in which maturing adults of the parent stock had apparently become widely dispersed in the Gulf before spawning (Fig. 5*A*), propagation of the first brood of stock *C* seems to have been largely if not entirely restricted east of Mt. Desert. By May 18–24 late season eggs were not found outside of the Bay of Fundy;[8] early larvæ occurred at but two out of seven stations in the western area (Fig. 6*A*), and late nauplius stages (Fig. 6*B*), although more widely dispersed, were centered in the Bay of Fundy and along the course of the coastal drift from the eastward. It is probable that these late nauplii originated in the eastern basin and not in the Bay of Fundy because in the latter area the largest numbers were found in the course of the entering drift. Indicating local conditions beyond the immediate influence of this inflow, there was little evidence of survival in New Brunswick and eastern Maine coastal waters (Fig. 6*B*).

An analysis of the dispersal and resulting regional abundance of subsequent generations of this stock is rather complicated. Apparently the large numbers of late nauplii entering the western area in May (Fig. 6*B*) failed to establish themselves, for when this brood matured and were spawning in late June (Fig. 2) the numbers of eggs everywhere west of Mt. Desert were relatively small and at several of the stations none were found (Fig. 7*A*). This may have been due to destruction by enemies as previously suggested (p. 208) because the rich local population of plankton animals was at its maximum, and, as shown by conditions in August (Fig. 8), all of the breeding stocks of *P. minutus* were rapidly depleted in that area. On the other hand, corresponding to this decline in the western Gulf, there was a surprising increase of adults and eggs in the Bay of Fundy in late June. In the New Brunswick area adult *P. minutus* formed up to 83.2 per cent of the total zoöplankton population (Table VI, p. 213) and eggs up to 33,975 per

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[8] Eggs, up to 1,405 per minute on May 19, were found only along the New Brunswick coast where the season appeared more retarded than elsewhere in the eastern region.

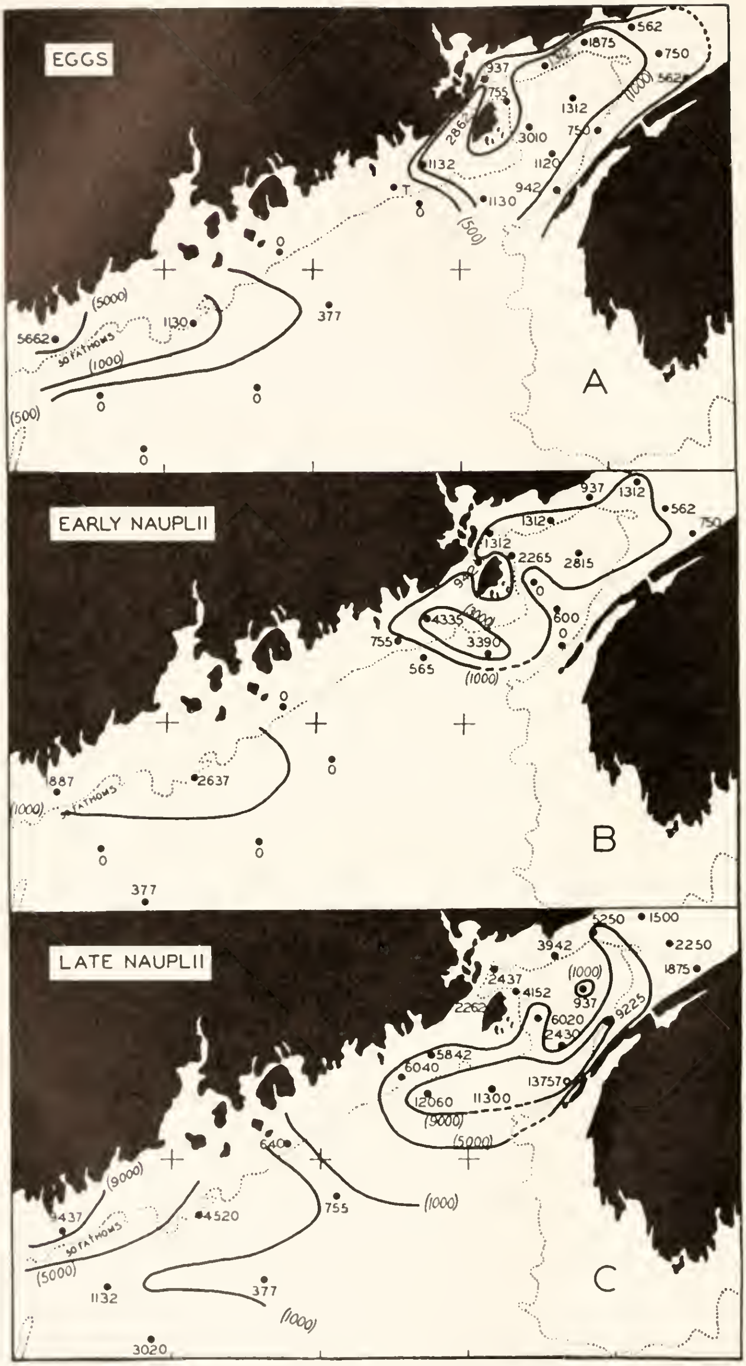
EGGS
562
1875
750
562
937
755
1312
(1000)
1312
2862
3010
750
1120
1132
942
1130
T.
0
(500)
0
0
377
(5000)
1130
5662
50 FATHOMS
(1000)
(500)
0
0
0
A
EARLY NAUPLII
937
1312
562
750
1312
1312
2265
2815
900
0
600
0
4335
(3000)
3390
755
565
(1000)
0
0
1887
2637
(1000)
50 FATHOMS
0
0
0
377
B
LATE NAUPLII
5250
1500
2250
3942
1875
(1000)
2437
4152
937
2262
6020
9225
2430
5842
6040
13757
12060
11300
(9000)
(5000)
640
(9000)
755
(1000)
3437
4520
50 FATHOMS
(5000)
1132
377
(1000)
3020
C

FIG. 8. Occurrence of eggs and early nauplii (*A–B*) of the third eastern brood and late nauplii (*C*) of the third western brood in August, 1932. Number per cubic meter.

cubic meter (Fig. 7*A*). The source of this population is somewhat uncertain, but, in view of the above-mentioned evidence of unsuccessful local production, it is believed that the adults consisted partly of members of the first brood of stock *C* from the eastern basin of the Gulf, and partly of the second brood of stock *A* from the outer Gulf (pp. 201–202). It is barely possible that propagation of this species is nowhere successful in the eastern Gulf and that all subsequent broods attributed to stock *C* were in reality migrants of stock *A*. This seems unlikely, however, since late nauplii off Mt. Desert in May (Fig. 6*B*) could hardly have been produced much farther away than the eastern basin.

By August further changes became evident. Eggs and early nauplii, presumably of the third brood of stock *C*, were concentrated in the Fundy region (Fig. 8*A* and *B*) and their scarcity or complete absence for some distance eastward from Mt. Desert would indicate that the depletion of the *P. minutus* population had now extended from the western Gulf to include the eastern basin. The similarity in the distribution of the two stocks, *B* (Fig. 8*C*) and *C* (Fig. 8*A* and *B*), in the Fundy region in August is most readily explainable on the basis that both were now centered in the same locality, and that the larvæ were derived from parents transported from the general vicinity of Georges Bank. Had observations been continued in September, it might have been possible to determine whether, with the decline of the predatory animal population, there occurred a progressive restocking to the westward in the inner Gulf by these migrants from offshore or their progeny. There is some indication that this may occur in Bigelow's (1926, p. 281) observation that there is "a greater absolute abundance over the area as a whole in late summer and autumn." Within the Bay of Fundy in mid-September, late nauplius stages of stock *C* (third brood) were most numerous (3,563 per cubic meter) in the entering drift from the Gulf and over the central basin.

Stock A.—Little can be said about the dispersal of this stock after April because of the probable confusion with stock *C* (p. 201). Originating offshore in March, by late April the larvæ, in late copepodite stages, were centered in the region of Georges and Browns Banks. Along the probable course of the drift smaller numbers had been dispersed to the outer part of the Bay of Fundy and along a tongue-like band penetrating the western area.

If copepodites in late June and August (p. 202) can be considered to consist in part of stock *A*, then this stock in the inner Gulf, at all times in the summer of 1932, remained restricted largely to the eastern basin and Bay of Fundy. One would have expected to find the progeny of the first brood (entering the western area in April) generally dispersed throughout the western Gulf, but the distribution of all copepodites in June (Fig. 9*A*) and again in mid-August (Fig. 9*B*) clearly indicated migration from the eastward.

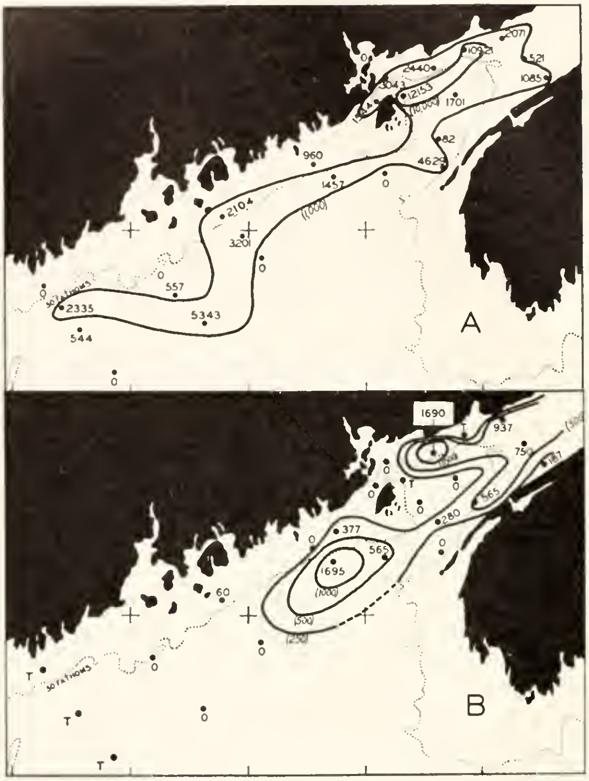
0
2071
10921
521
2440
1085
3043
12153
1701
(10,000)
82
960
462
1457
0
2104
(1,000)
3201
0
0
557
50 FATHOMS
0
2335
5343
544
0
A
1690
937
75
187
(500)
T
0
0
565
280
0
377
0
565
1695
(1000)
60
(500)
(250)
0
T
50 FATHOMS
0
T
0
T
B

Fig. 9. Occurrence of late copepodite stages considered indicative of the outer Gulf stock in June (*A*) and August (*B*), 1932. June: number per minute of towing. August: number per cubic meter.

Relative Numerical Strength of Pseudocalanus minutus (Adults) in the Zoöplankton Population

In the Gulf of Maine and Bay of Fundy *Pseudocalanus minutus* is second only in importance to *Calanus finmarchicus*. Offshore it is usually far outnumbered by the latter species, but in inshore waters such as the Quoddy region (Sta. 5, Table VI) and bays in the Gulf it is at times the dominant form. A similar condition is also found in the coastal region south of Cape Cod (Fish, 1925, p. 144, Fig. 45), and to the northward in the Gulf of St. Lawrence where Willey (1919, p. 187) found it constituting between 80 and 90 per cent of the copepod content between Prince Edward Island and the Magdalen Islands in May, 1915.

Combining the results of several years in the Gulf of Maine, Bigelow (1926, p. 282) found that *Pseudocalanus* averaged about 11 per cent of the copepod stock and as a rule was from one-tenth to one-fifth as abundant as *Calanus.* In 1932 during the period from April until September *Pseudocalanus* averaged 4.9 per cent of the total zoöplankton population of the Gulf, and *Calanus* 42.9 per cent, a ratio falling within Bigelow's range.

In the Bay of Fundy, based on monthly averages for three stations, the mean percentage of *Pseudocalanus* in meter net hauls for the year ending in August, 1932 was approximately 10, as shown in Table VI.

TABLE VI

1931–1932	*Pseudocalanus minutus*			
Month	Mean	Sta. 5	Sta. 6	Sta. 8A
September	2.3	3.7	2.8	0.5
October	1.5	1.5	——	——
November	1.9	2.1	3.3	0.3
December	3.1	4.5	2.3	2.5
January	8.2	8.2	——	——
March	12.0	6.6	10.9	18.5
April	5.8	5.5	5.8	6.0
May	22.0	53.2	8.0	4.8
June	43.4	83.2	45.2	1.7
July	3.0	3.0	——	——
August	5.3	15.2	0.8	0
Mean for Year	9.9	17.0	9.9	4.3

Along the New Brunswick shore (Station 5) it formed a much more important part of the plankton population than over the central basin or along the Nova Scotian side. Whereas *Calanus* increased in a seaward direction from the New Brunswick coast, the relative percentages of *Pseudocalanus* for the year decreased from 17 per cent in the Quoddy region to 4.3 per cent at the entrance to the Bay (Station 8*A*). This condition remained constant except for a short period following vernal augmentation when the percentage was greatest in the path of the drift from the Gulf. The highest value in the Bay (83.2 per cent) was found in the outer Quoddy region in June.

During the summer (April–September) the relative percentage of *Pseudocalanus* decreased westward from the Bay of Fundy, averaging 17.2 per cent in the Bay and as previously mentioned, 4.9 per cent in the Gulf, with minimum values in the western area.

Monthly averages indicated a definite seasonal variation in all areas covered in 1932 as the summer advanced. The highest value for the western area (10.6 per cent) was in April, for the central area (12.5 per cent) a month later in May, and for the New Brunswick area (59.2 per cent) in June. These results reflect the progressive response to vernal augmentation eastward along the coast. Indicating immigration from the western and outer Gulf, the Nova Scotian area yielded the highest percentages in March and April. Almost everywhere there was a continued decline after the first maximum, in spite of subsequent propagation.

The importance of *P. minutus* in the natural economy of the region is obviously greater than the relative numerical strength of the adult population would imply. Although, as Bigelow (1926, p. 282) has pointed out, the adult stock is still far behind *Calanus* because of its smaller size and fewer numbers, the enormous number of larvæ produced and subsequent rate of depletion indicate that its importance to those small predatory forms (including several of the more important species of Gulf fishes during their early existence) feeding on larval copepods must be considerably greater in some parts of the region than that of *Calanus*.

Table VII

1932			Gulf of Maine			Bay of Fundy		
Cruise	Month	Total region	Total gulf	Western area	Central area	Total bay	New Brunswick area	Nova Scotian area
26	April	12.4	7.9	10.6	5.2	21.5	29.0	14.4
27	May	12.7	8.1	4.3	12.5	21.4	34.3	6.4
28	June	16.8	7.3	3.5	11.7	35.3	59.2	7.5
30	August	2.1	1.0	0.7	1.4	4.3	4.8	3.7
32	Sept.	1.3	0.1	0.3	T	3.6	2.1	5.1
Mean	April–Sept.	9.1	4.9	3.9	6.2	17.2	25.9	7.4

Summary

1. *Pseudocalanus minutus* is generally distributed both in neritic and offshore waters. Having a somewhat greater thermal range it regularly outnumbers *Calanus* in neritic areas south of Cape Cod and on occasions in boreal waters also.

2. Propagation begins in March and continues in a succession of generations until September. It starts earlier in the southern part of the range where there are also a greater number of annual broods.

3. Eggs appearing at progressively later periods (approximately monthly intervals) in the outer Gulf, western area, and Bay of Fundy make possible a distinction of three major breeding stocks.

4. In the western stock there are at least three and possibly four breeding periods, March–April, May–June, July–August and September.

5. In the eastern stock there is evidence of three breeding periods, April–May, June–July, and August.

6. The outer Gulf stock is not clearly traceable due to probable overlapping of subsequent breeding periods with those of the eastern stock, but four periods are indicated.

7. There appears to be a developmental period of approximately two months throughout the region.

8. *Pseudocalanus* is very prolific and its early larvæ greatly outnumber those of *Calanus.* Mortality is relatively much higher in *Pseudocalanus,* the depletion being greatest in nauplius stages, although a second critical period during maturation is indicated.

9. There is no evidence of successful propagation in the Bay of Fundy, and the western area declines in importance after the first brood. Subsequent propagation of all breeding stocks appears to be centered in the outer Gulf.

10. The adult stock of *Pseudocalanus* is second only to that of *Calanus* in numerical importance, averaging 9.9 per cent of the total zoöplankton population in the Bay of Fundy during the year 1931–32 and 4.9 per cent of the summer population in the Gulf.

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DIURNAL MOVEMENTS OF THE EYE PIGMENTS OF ANCHISTIOIDES

JOHN H. WELSH

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I

Physiological rhythms which have been established in organisms by regular environmental changes, such as the change in light intensity between day and night, occasionally persist when the external recurring stimuli are removed and the organism is kept under constant conditions. An example of such a phenomenon is seen in the movements of the eye pigments of certain crustaceans. The distal pigment cells in the eye of *Macrobrachium* continue to move back and forth along the ommatidia when animals are kept under constant illumination (Welsh, 1930*b*). The proximal pigment in the eye of *Cambarus* shows a partial migration, with periods corresponding with day and night, when animals are kept in constant darkness (Bennitt, 1932). Several species of Bermuda crustaceans show diurnal changes in one or more of the three sets of pigments of the eye when kept in light of a constant intensity or in constant darkness (Welsh, 1935).

When regularly recurring variations in external conditions are prevented, as they may readily be in the case of aquatic organisms, and the organism still responds as though in its normal environment, it is possible to account for the response only on the basis of an internal controlling mechanism. In the case of a diurnal rhythm the response is normally due to changing light intensity, temperature, humidity, or some other factor which varies with day and night. The response might be due entirely to the direct effect of one or more of these factors but if it persists under constant conditions it is evident that it is more complex than this and is probably effected by way of the nervous system or a combination of nervous and endocrine systems.

It would appear to be of rather general interest to have more detailed information on the nature of the controlling mechanism and the crustacean material is quite satisfactory for such an investigation. One fact that must be known in the case of the changes in the crustacean eye is how long the rhythm persists. The present paper is concerned primarily with this problem.

The experimental work was carried on in Bermuda and was in part made possible by a grant from the James F. Porter Fund. The author is indebted to Dr. J. F. G. Wheeler, Director of the Bermuda Biological Station, for his kind coöperation.

II

In 1933 two specimens of an unusual shrimp were taken in Bermuda by the author while collecting at night. In 1934 Dr. F. A. Brown obtained many more specimens which eventually proved to be *Anchistioides antiguensis* Schmidt, a form rarely taken in the Atlantic Ocean. Brown and Wheeler have been able to show (results not yet published) that this form is active only at night and can be collected in numbers only on the dark of the moon, thus showing a lunar periodicity. Because of its transparency, no pigment being present except in the eyes, it was selected for the present work. Preliminary observations on the eyes of animals kept in light of constant intensity or in constant darkness, except for short periods of examination, indicated slight diurnal changes in the distal pigment of the eye in the light, and complete normal daily movements in darkness. The appearance of such eyes when observed microscopically by means of transmitted light may be seen in Fig. 1, *A–D*. The condition seen in the eye of an animal kept in constant illumination and observed during the day will for convenience be called the " day-light " condition and may be seen in Fig. 1*A*. The same eye at night shows the distal boundary of the pigment nearer the periphery of the eye and this will be called the " night-light " condition (Fig. 1*B*). An animal removed from the dark during the daytime, and observed immediately, shows the distal pigment in the same position as is seen in the light during the day. This will be called a " day-dark " eye (Fig. 1*C*). If the same animal is returned to the dark and an examination made at night the entire eye is seen to be black except for a very thin border representing the cornea (Fig. 1*D*). This will be called the " night-dark " condition.

Observations of the intact eye show clearly the changes in position of the distal pigment cells and the changes which occur in the caps of reflecting pigment on the distal pigment cells, but changes in the main mass of reflecting pigment and in the proximal pigment can be seen only in sections of the eye.

The eye of *Anchistioides* as seen in sections is essentially like that of *Palæmonetes* which has been described by Parker (1897) and Welsh (1930*a*). The pigment changes are similar in most respects to those of *Palæmonetes* as described in these papers and may be compared with other crustaceans by reference to the review by Parker (1932).

Material for histological study was prepared in the following manner. A number of animals which had been caught the previous night were distributed among four containers. Two were illuminated with light from a 40W lamp at a distance of eighteen inches. Two were placed in a dark room. The period of adaptation began at 9:00 A.M. At 3:00 P.M. one lot was removed from the light and one from the dark and immediately fixed in hot water at 80° C. The heads were then cut off and placed in 70 per cent alcohol. At 11:00 P.M. the remaining two lots were treated in a similar manner. The eyes of a given lot of animals after sectioning showed the pigments in similar positions. Plate 1, Fig. 3–6, show sketches of ommatidia based on camera lucida drawings. The situation is rather complex and each set of pigment must be followed through the series in order to make clear the changes which result from changes in light intensity and those which occur under constant conditions. The following summary supplements the figures and applies to the eye as a whole.

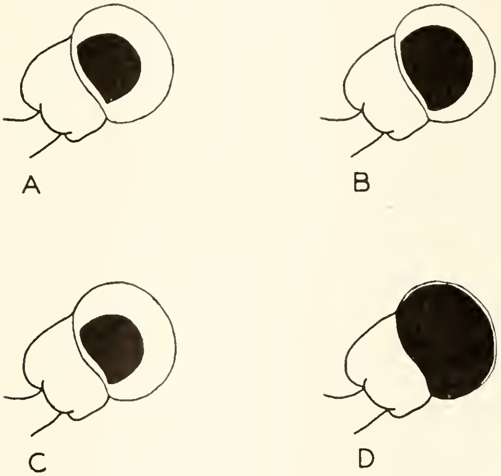

FIG. 1. *A*. Eye of an animal kept in constant illumination showing the position of the distal pigment cells during the day. A "day-light" eye.

B. Same eye as in *A* viewed at night. A "night-light" eye.

C. Eye of an animal kept in constant darkness except for short observational periods showing the position of the distal pigment cells during the day. A "day-dark" eye.

D. Same eye as in *C* viewed at night. A "night-dark" eye.

Day-light Eye. Fig. 3.

Distal Pigment Cells—In extreme proximal position resting on the retinular cells.

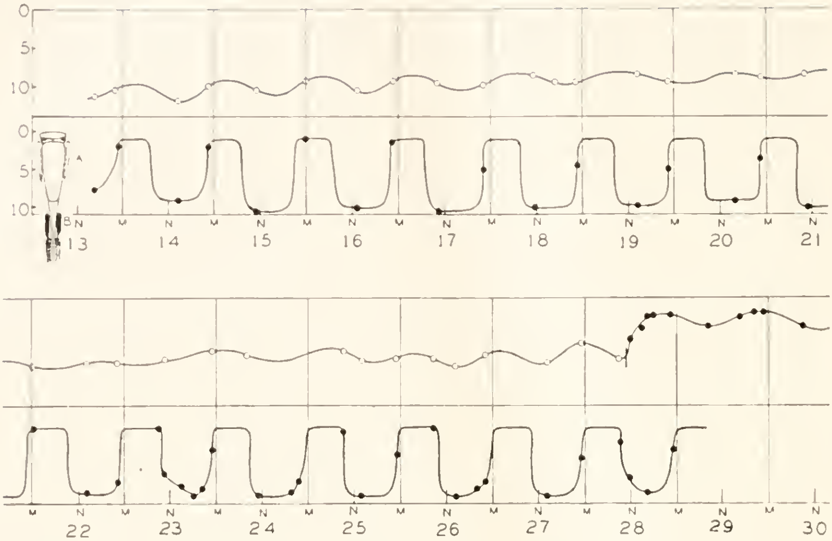

FIG. 2*A*. Records of the movements of the distal pigment cells of four animals kept in constant illumination (open circles), except for last two days, and of four animals kept in constant darkness (solid circles). Experiment started June 13 and ended on June 30. Noon and midnight are indicated by the letters *N* and *M*.

In the sketch of an ommatidium *A* is the extreme peripheral or night-position of the distal pigment cells, *B* the inner or day-position, in constant darkness.

The points as plotted are averages of the measurements of the distance from cornea to outer boundary of pigment. Each unit = 10 microns.

Time of sunset during course of the experiment about 7:35 P. M.

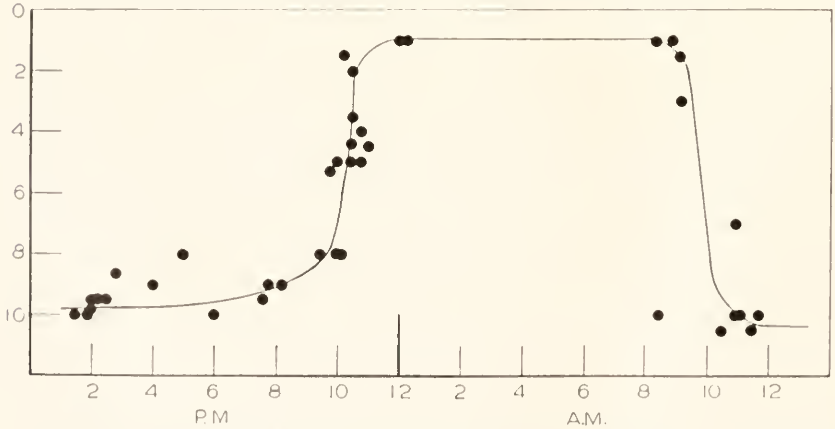

FIG. 2*B*. The measurements made on the eyes of animals kept in constant darkness and shown plotted over a period of fifteen days in Fig. 2*A* are here shown in one 24-hour period. Such a plot makes it possible to construct a curve representing the daily movements of the distal pigment cells. One of the coordinates shows the hours of the day beginning at noon, the other the distance of the distal pigment cells from the outer boundary of the cornea, each unit being equal to 10 microns.

Proximal Pigment—In retinular cells concentrated around rhabdomes. Some beneath the basement membrane.

Reflecting Pigment—Large masses resting on distal pigment cells, the remainder equally distributed above and below the basement membrane.

Night-light Eye. Fig. 4.

Distal Pigment Cells—Elongated but with proximal ends still in contact with retinular cells.

Proximal Pigment—Distributed as in day-light condition.

Reflecting Pigment—Most of this pigment which capped the distal pigment cells in the day-light conditions has moved proximally and fused with the mass below the basement membrane.

Day-dark Eye. Fig. 5.

Distal Pigment Cells—As in day-light condition.

Proximal Pigment—Above and below basement membrane. Very little in retinular cells.

Reflecting Pigment—Main mass around bases of retinular cells. Small caps on distal pigment cells and small masses below basement membrane.

Night-dark Eye. Fig. 6.

Distal Pigment Cells—In extreme distal position forming a collar around cones.

Proximal Pigment—Main mass below the basement membrane, none in distal part of retinular cells.

Reflecting Pigment—All is massed about the bases of the retinular cells where it forms a reflecting surface sending light back into the rhabdomes.

It is evident that the most striking changes in the eye which are independent of changes in the environment are seen when animals are kept in constant darkness. During the day the distal pigment cells are in the extreme inner position and during the night they move to the extreme outer position. In constant illumination, of the intensity used, the diurnal movement is not so striking as no change in position occurs, but only a change in length of the distal pigment cells.

It is not necessary to discuss further the changes in the reflecting pigment as they are clearly shown in the figures. The behavior of the reflecting pigment in certain other forms has been described elsewhere (Welsh, 1932). In this form the proximal pigment exhibits only slight changes which are independent of light and in this respect is like *Macrobrachium*.

In the case of *Macrobrachium* it had already been found (Welsh, 1930*b*) that the distal pigment cells continued their characteristic movements when the animals were constantly illuminated, and it was possible to follow closely the time relations and determine that the outward movement began at the time of sunset and the inward movement at the time of sunrise. Similar observations were made on *Anchistioides* kept in the light and in the dark. On June 13 four animals in separate bowls of sea water were placed in the dark and four were placed in a situation where they received only light from a 40W lamp. During the course of the experiment they were not fed but the water was changed once or twice each day. They were usually examined once near midday and once at about 11:00 P.M. The distance from the cornea to the periphery of the central pigment mass was then measured by means of an ocular micrometer. In the case of the animals kept in the dark this meant a period of illumination of a few minutes twice in twenty-four hours. Measurements were made for sixteen days when the experiment in the dark was discontinued as two of the animals died on June 29. On June 28 the animals which had been in the light for fifteen days were transferred to the dark and measurements continued for two days and then stopped as our stay in Bermuda was at an end.

The results are shown graphically in Fig. 2*A*. The points as plotted are averages of the measurements on the four animals. Although there were individual variations they were not great and each animal responded essentially as the group as a whole. In the dark the movement of pigment was as extensive at the end of the experiment as at the beginning, and the only change was a slight lag which may be seen by following the positions of the pigment at 10:30 or 11:00 P.M. when measurements were usually made. If all of the measurements made on the eyes of

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EXPLANATION OF PLATE I

Ommatidia (270 ×) showing details of pigment distribution.
Sketches are based on camera lucida drawings.

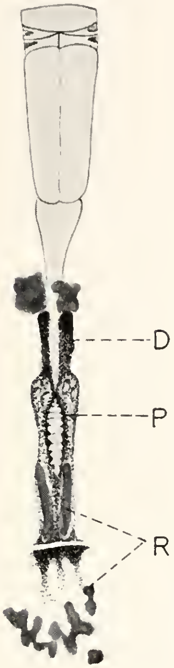
Fig. 3. Ommatidium from an eye of an animal constantly illuminated and killed and fixed during the day. "Day-light" condition.

Fig. 4. Ommatidium from an eye of an animal constantly illuminated and killed and fixed during the night. "Night-light" condition.

Fig. 5. Ommatidium from an eye of an animal kept in constant darkness and killed and fixed during the day. "Day-dark" condition.

Fig. 6. Ommatidium from an eye of an animal kept in constant darkness and killed and fixed during the night. "Night-dark" condition.

D = distal pigment; *P* = proximal pigment; *R* = reflecting pigment (the masses of reflecting pigment which rest on the distal pigment cells are not labelled).



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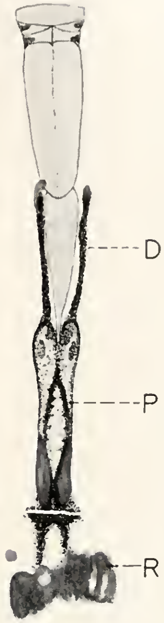

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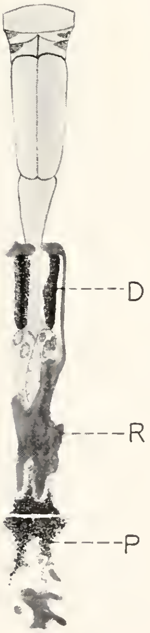

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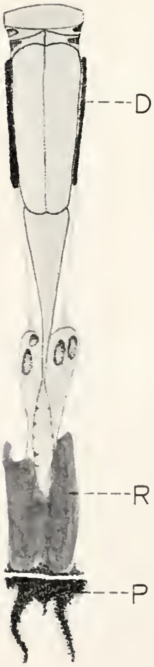

6

Plate I

animals kept in constant darkness are plotted in one 24-hour period, as seen in Fig. 2*B*, it is possible to draw a curve which represents quite accurately the movements of the distal pigment cells. The outward movement of the pigment which begins at about the time of sunset is slower than the inward movement occurring in the morning. This is in agreement with the results obtained in a study of the rate of migration of the distal pigment cells in the eye of *Palæmonetes* (Welsh, 1930*a*), where it was found that distal migration took approximately twice as long as proximal migration. The curve obtained here may now be applied to the data as shown in Fig. 2*A* and justifies the curves which are shown in this figure.

As has already been pointed out, the changes in position of the distal pigment cells, in constant illumination of the intensity used in this instance was not great; nevertheless it is possible to follow their rhythmical migration, which, at least for a few days, kept in phase with day and night. On June 17 and 18 an apparent lag is noticeable and on June 20, 21, and 22 the pigment cells seem to be out of phase with day and night. During this period the changes in position of the pigment cells were very slight and it is quite possible that this is an incorrect interpretation as later on June 25, 26, and 27 they are again in phase. A rhythm approximating a 24-hour one was present, however, and continued throughout the experiment. Some change resulted from the prolonged exposure to light for when the animals were placed in the dark the movement was not as extensive as in those animals which had been kept in constant darkness. It is probable that longer stay in the dark would have resulted eventually in a return to a more normal condition. It is also possible, although the experiment was not tried, that constant illumination of a higher intensity than that used would have completely suppressed the movements of the distal pigment cells.

III

The persistence of a rhythm in the movements of the eye pigments of *Anchistioides* for a period of more than two weeks indicates that it is no transitory phenomenon with which we are dealing. The mechanism which is responsible for the changes in the eye continues its cyclic activity long after all recurring external stimuli are removed. It is highly unlikely that the rhythm is a property of the pigment cells. That it is a property of one of the main coördinating systems, either nervous or endocrine, is more nearly possible. Some of the evidence to support this conclusion will be considered briefly. In the earlier work on *Macrobrachium* it was suggested that both the nervous and endocrine systems were involved in the diurnal rhythm in the eye of this form. The results from the use of anaesthetics and the ligation of the eye-stalk led to this conclusion. The immediate control appeared to be hormonal yet somewhere in the chain the nervous system quite evidently played a part. At that time much less was known concerning the control of pigment migration in the crustacean eye than at the present time. Since then Kleinholz (1934, and in more recent unpublished work) has demonstrated, in a wide variety of decapod crustaceans, the part played by the so-called eye-stalk hormone in the activity of the eye pigments. It was reported (Welsh, 1935) that Kleinholz had found the eye-stalk extract controlled the inward movement of only the distal pigment cells. Since then the reflecting pigment has also been shown to be under the influence of material from the eye-stalk. No hormonal effect on the proximal pigment has yet been demonstrated. It is interesting to note that in *Anchistioides* the persisting movements are most extensive in the distal and reflecting pigments.

The work of Hanström (1931, 1934, 1935) and certain of his students (Sjögren, 1934; Carlson, 1935) has done much to further our knowledge of the sources of the eye-stalk hormone or hormones. These investigators have described two glands generally present in the crustacean eye-stalk which are quite certainly endocrine in nature. One they call the blood gland and the other the X-organ. Carlson has shown that the blood gland in *Uca* is the source of at least one of the pigment-concentrating substances affecting body chromatophores. The function of the X-organ still remains in doubt. These glands are supplied with nerves and their activities are doubtless controlled in part by impulses from the eyes arriving by way of the brain.

It was fortunately possible to have Dr. Hanström examine the eye-stalks of *Anchistioides* and he reported the presence of both the blood gland and the X-organ. The blood gland in this form is normally developed and is lying between the second and third optic ganglia on the dorsal side of the nerve mass. The X-organ is very well developed and is unusually far distal, almost touching the basal membrane of the eye. As in other decapods, its position is in the ventral part of the eye-stalk.

The hormone producing the movement, characteristic of the light, of the distal and reflecting pigments doubtless comes from one of these glands of the stalk. Normally we can assume that it is released into the blood stream under the stimulus of illumination, thence it is carried to the eye to produce so-called light adaptation of the eye. Under constant illumination in *Anchistioides* the production and secretion of the hormone continues and the pigment remains in a nearly constant light position depending on the intensity of illumination. In constant darkness, however, the situation is not so obvious. Why should the hormone be released during daylight hours and not during the hours of darkness and why should this process continue for two weeks without becoming irregular or showing signs of disappearing? At present it is only possible to speculate. There may be a rhythmic secretory cycle in the gland which continues under constant conditions or the situation may be much more complex and the rhythm in the eye may only accompany a general rhythmic activity which results from a series of changes involving the nervous-endocrine systems. Experiments which are now being carried on may throw further light on this interesting phenomenon.

SUMMARY

1. The distal pigment cells and the reflecting pigment in the eyes of *Anchistioides*, a crustacean, continue their characteristic movements under constant external conditions.

2. The movements of the distal pigment cells continued for fifteen days in animals kept in constant darkness and in constant illumination. Movements, normal in extent, occurred in constant darkness while in constant illumination the movements were greatly suppressed.

3. The persistence of this diurnal rhythm indicates that an internal cyclical mechanism is responsible, in part, for pigment movements in crustacean eyes.

4. The possibility of the blood gland or X-organ being concerned in the diurnal rhythm in retinal pigment movement is considered.

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RENAL FUNCTION IN MARINE TELEOSTS

III. The Excretion of Urea [1]

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The manner in which urea is handled by the kidney shows wide variations in the group of vertebrates. There is excellent evidence that urea is normally reabsorbed from the glomerular filtrate in considerable amounts by the renal tubules of the dog (Jolliffe, Shannon, and Smith, 1932; Shannon, 1935*a*; Van Slyke, Hiller, and Miller, 1935*a*, 1935*b*) and man (Shannon, 1935*b*; Shannon and Smith, 1935). Walker and Elsom (1931) have demonstrated that urea is freely filterable across the glomerular membrane of the frog, and conclude that " it is not necessary to assume tubular secretion of urea to account for the renal elimination of this substance in the frog." Marshall (1932), on the other hand, from xylose-urea comparisons, has concluded that urea may be secreted in large amounts by the renal tubules of the frog.[2]

For the marine teleosts the data are rather fragmentary. Marshall and Grafflin (1932), on the basis of glucose-urea urine/plasma ratios under phlorizin, were led to the conclusion that in the glomerular sculpin (*Myoxocephalus octodecimspinosus*) urea can be secreted by the renal tubule. Their experiments upon the aglomerular toadfish (*Opsanus tau*) indicated that urea is normally present in the urine in essentially the same concentration as exists in the plasma.[3] The striking observation was made that the sculpin kidney can be rendered functionally aglomerular by the administration of large doses of phlorizin; and under these conditions the urine/plasma urea ratio approaches the 1 : 1 value characteristic of the aglomerular toadfish. The aglomerular goosefish (*Lophius piscatorius*) is unique among the marine teleosts so far examined in that both plasma and urine consistently contain urea in only minimal amounts (see discussion below).[4] A few data upon urinary urea in *Gadus callarias* and *Sphcroides maculatus* (both glomerular—Nash, 1931) have been reported by Smith (1929).

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[1] The first two papers of this series appeared in the *Biological Bulletin*, Vol. 69, p. 391, and Vol. 70, p. 16.

[2] Even admitting that xylose gives values for the glomerular filtrate which are too low (see discussion below), it is hardly conceivable, in the light of our present knowledge, that Marshall's results could be explained on the basis of glomerular filtration alone.

[3] Bieter (1931) had previously demonstrated that urea exerts a marked diuretic effect upon the aglomerular kidney of this species.

For the catfish, a fresh water teleost, Marshall (1932) reports finding urea concentration ratios lower than those of xylose, indicating a reabsorption of urea by the renal tubule.[5] In the marine elasmobranchs, which show a high degree of specialization in the tremendous urea content of their blood and tissues (Smith, 1932), the urea filtered at the glomerulus is almost completely reabsorbed by the renal tubules (Clarke and Smith, 1932). From the data available (Smith, 1931), this is likewise true in the case of the fresh water elasmobranchs, in which the blood urea level is much lower than in the marine forms.

The data reported in the present paper deal primarily with urine/plasma urea concentration ratios in the glomerular marine teleosts. Since this group of fishes normally shows a rather low rate of urine flow, but develops a marked diuresis under the usual experimental conditions (Grafflin, 1931, 1935; Pitts, 1934; Grafflin and Ennis, 1934; Clarke, 1934), the ability of the kidney to concentrate urea at widely varying urine flows has been investigated, particularly in the sculpin and to a lesser extent in the flounder. In addition, a few experiments are reported upon the behavior toward injected urea of the aglomerular kidney of the goosefish. This study was carried out at the Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine.

MATERIAL AND METHODS

The animals used in this investigation were the sculpin (*Myoxocephalus octodecimspinosus*), flounder (*Pseudopleuronectes americanus*), daddy sculpin (*M. scorpius*), sea raven (*Hemitrypterus americanus*), wrymouth (*Cryptocanthodes maculatus*) and goosefish (*Lophius piscatorius*). The rate of urine flow was determined in all species but the goosefish in the following manner: the bladder was first emptied, with the use of a fine glass catheter, by massage of the overlying abdominal wall; the urinary papilla was then tied off with fine silk thread, and at the end of the collection period the urine was removed from the exposed bladder by syringe. The length of the collection period was variable: sculpin, 3 to 24 hours; flounder, 5 to 23 hours; daddy sculpin, 18 to 23 hours; sea raven, 18 hours; wrymouth, 7 hours. Many of the fish were tied up immediately after catching, others only after a variable delay; and some were deliberately rendered diuretic by rough handling. All animals were unfed after catching, and the effect of starvation for a moderate length of time was studied especially upon six sculpins, which are marked with an asterisk in Table I. These fish were caught and handled throughout with every precaution, and were starved in laboratory aquaria for twelve days before the collection period was begun.

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[4] The data reported for the goosefish by Edwards and Condorelli (1928) cast doubt upon all of their urea analyses (see below), and their blood and urine figures for *Syngnathus*, *Hippocampus*, and *Muræna* will not be further discussed.

[5] Smith (1929) gives urinary urea data, without simultaneous blood analyses, for many species of fresh water teleosts.

Sculpin, daddy sculpin, sea raven, and wrymouth were bled from the exposed heart by syringe, flounder from the severed caudal vessels. Bloods were drawn at the end of the collection period, rather than at the beginning and end or in the mid-period. The procedure used is perhaps to be preferred in view of Clarke's (1934) observation that a single bleeding in the course of an experiment may result in a marked interference with urine excretion. Marshall and Grafflin (1932) reported that plasma urea in the sculpin showed little variation in the course of twenty-four hours. However, a not inconsiderable variation was found in the toadfish in some experiments, and such a variation almost certainly occurred in many of the animals reported here. Since, from the toadfish results, the variation would presumably be in both directions, a group of animals would yield acceptable results, even though individual U/P ratios might not be accurate.

Goosefish were bled by syringe from the unexposed heart, and the bladder was emptied and urine collected by the use of a suitable catheter and massage of the overlying abdominal wall.

Urea was determined by the urease method in the Van Slyke apparatus (Van Slyke, 1927), with the use of 0.5 M veronal, rather than phosphate, as buffer because of the high magnesium content of the urine. All urea analyses with rare exceptions were done in duplicate, with the samples exposed to urease for three and six minutes. Heparin was used throughout as anticoagulant.

RESULTS

Sculpin

The data on urea excretion in the sculpin are summarized in Table I, and demonstrate the following facts: (1) there is a considerable variation in the plasma urea level (5.3 to 18.7 mgm. per cent, with one value of 30.9 mgm. per cent); (2) the sculpin kidney is able to concentrate urea at all observed plasma urea levels and levels of urine flow, the degree of concentration being less in the higher range of flow (flow 3.7 to 15.2 cc., average U/P ratio 2.1; flow 17.4 to 52.0 cc., average U/P ratio 1.5); (3) starvation for twelve days (animals marked with an asterisk in Table I) does not cause a detectable alteration in the plasma urea level (7.7 to 18.3 mgm. per cent) or in urea concentration by the kidney (flow 7.5 to 17.4 cc., average U/P ratio 1.9).[6]

The U/P ratios for urea become of particular interest when compared with Clarke's (1934) very careful study of the excretion of xylose, which he used, on the basis of previous work (Jolliffe, Shannon, and Smith, 1932; Clarke and Smith, 1932), as a measure of glomerular filtrate. His conclusion was that in the sculpin "with progressive diuresis there is a tendency for the increased urine flow to be effected by increasing glomerular filtration with a constant reabsorption of water

TABLE I

Renal excretion of urea in marine teleosts

SCULPIN				FLOUNDER			
Urea, mgm. per cent		U/P ratio	Urine flow	Urea, mgm. per cent		U/P ratio	Urine flow
Plasma	Urine			Plasma	Urine		
			cc. per kg. per 24 hrs.				*cc. per kg. per 24 hrs.*
14.6	24.6	1.6	3.7	13.3	23.3	1.8	8.3
30.9	62.2	2.0	7.5	11.2	10.9	1.0	10.3
13.3 *	24.4	1.8	7.5	9.3	11.6	1.2	10.4
12.7	32.8	2.6	7.8	14.4	23.0	1.6	13.0
10.5 *	14.4	1.4	7.8	8.5	14.0	1.6	13.2
10.8	19.5	1.8	8.3	18.4	25.9	1.4	18.9
11.0 *	27.6	2.5	9.3	11.3	15.6	1.4	26.4
7.7 *	17.2	2.2	9.5	12.1	13.2	1.1	26.7
4.6	9.1	2.0	10.2	10.1	12.8	1.2	37.0
9.5	18.2	1.9	10.5	12.3	11.7	1.0	43.0
5.5	16.1	2.9	10.7	11.3	13.6	1.2	45.6
18.3 *	34.1	1.9	11.0				
14.1	34.7	2.5	11.2	DADDY SCULPIN			
13.1	24.4	1.9	11.9				
15.6	44.0	2.8	15.2	14.0	19.6	1.4	3.2
10.7 *	18.9	1.8	17.4	7.8	8.5	1.1	10.5
6.7	7.9	1.2	18.2	10.2	10.2	1.0	37.6
5.3	7.8	1.5	19.2				
16.6	23.8	1.4	20.3	SEA RAVEN			
12.7	18.5	1.5	22.3				
18.7	28.8	1.5	31.7	8.2	7.3	0.9	8.3
11.9	17.6	1.5	44.5				
13.9	21.3	1.5	45.9	WRYMOUTH			
13.1	19.0	1.5	51.9				
14.8	16.7	1.1	52.0	6.7	22.1	3.3	11.3

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[6] For a description of the structure of the sculpin nephron, see Defrise (1932) and Edwards (1935).

(xylose U/P = 4) until the urine flow exceeds 10 cc. per kilogram per day; further diuresis is effected by a further increase in glomerular filtrate accompanied by a fall in the U/P ratio toward 1.0." More recent work (Richards, Westfall, and Bott, 1934; Shannon, 1934, 1935*a*, 1935*b*; Shannon and Smith, 1935; Van Slyke, Hiller, and Miller, 1935*a*) has led to the conclusion that xylose is reabsorbed to a considerable extent by the renal tubules, so that Clarke's figures for the glomerular filtrate must be considered to be too low. It is obviously unjustifiable for the present to draw any rigid conclusions from a comparison of Clarke's xylose data and ours on urea. However, the suggestions obtained from such a comparison merit consideration; namely, (1) that in the sculpin glomerular filtration alone seems adequate to account for all of the urea excreted; and (2) (and this applies primarily to the lower, more normal range of urine flow) that urea may be reabsorbed by the renal tubules in considerable amounts. In the light of our present knowledge, Marshall and Grafflin's (1932) earlier interpretation that urea may normally be secreted by the sculpin tubule is considered to be untenable without further proof. In view of the fact that a retention of nitrogenous metabolic products has been postulated to be of marked significance in the broad problem of osmotic regulation in the marine teleosts, and to be a necessary condition for the formation of trimethylamine oxide (Hoppe-Seyler, 1930*a*, 1930*b*; for discussion see Grafflin and Gould, 1936), it is very desirable that a further study of the excretion of urea and trimethylamine oxide in this group of fishes should be carried out simultaneously with an accurate measurement of the glomerular filtrate. At the present time inulin seems to be the substance of choice for this purpose.[7]

Flounder

The data upon flounder (kidney glomerular—Nash, 1931) indicate the ability of the kidney to concentrate urea, and it is noteworthy that the highest U/P ratios fall in the lower range of urine flow. Since no observations upon the measurement of glomerular filtration in the flounder are available for comparison, the probable mechanism of urea excretion in this species will not be discussed at this time.

Sea Raven, Wrymouth, Daddy Sculpin

The figures on single specimens of sea raven and wrymouth (kidneys of both species glomerular—Nash, 1931) are submitted without further comment. In regard to the daddy sculpin, it is interesting that the kidney of this species shows marked evidences of glomerular degeneration, so that some specimens are functionally entirely aglomerular (Grafflin, 1933). The fishes reported were of approximately the same weight (550–660 grams), but unfortunately no check upon their glomerular status was obtained.

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[7] For a discussion of the excretion of urea and ammonia by the gills of fishes, see Smith (1929).

Goosefish

In sharp contrast to the findings of Denis (1912–13; 1913–14) and Edwards and Condorelli (1928), who reported the presence of moderate to large amounts of urea in the blood and urine of the goosefish, was the observation of Marshall and Grafflin (1928) that they were unable to find more than a trace of urea in either the blood or the urine of this fish: plasma (or serum) 0.3 to 0.7, urine 0.2 to 0.5 millimols per liter. This observation was confirmed for the urine by Grollman (1929), who in four composite urine samples found the urea nitrogen to vary from 0.4 to 1.5 mgm. per cent (total nitrogen 56 to 373 mgm. per cent); and by Smith (1929), who in eight specimens found urea nitrogen values varying from 0.5 to 2.1 mgm. per cent (total nitrogen 31 to 60 mgm. per cent). The urea analyses of Marshall and Grafflin and of Grollman were carried out by the urease method in the Van Slyke apparatus (Van Slyke, 1927), those of Smith by decomposition with urease after extraction of ammonia with permutit; and it seems entirely reasonable to ascribe the results of Denis and of Edwards and Condorelli to the non-specificity of the methods employed.[8]

The goosefish is of particular interest because it is the only marine teleost so far reported in which urea is consistently present in the blood and urine in such minimal amounts. The kidney of this species is composed of large numbers of aglomerular tubules, but contains a relatively small number of glomerular structures (Edwards, 1928; Marshall and Grafflin, 1928), which Grafflin (1929) has shown to be incapable of function in the adult. That the aglomerular condition *per se* bears no essential relation to the urea findings in goosefish is indicated by the results obtained upon the aglomerular toadfish (Marshall and Grafflin, 1932).

Plasma-urine comparisons in the toadfish furthermore indicated that urea is present in the urine in approximately the same concentration as exists in the plasma. This finding led to the present experiments, designed to study the behavior of the aglomerular kidney of the goosefish toward injected urea. The low plasma and urine urea levels before in-

[8] Denis (1912–13), method of Folin (1912); Denis (1913–14), presumably the same method for urine, and the method of Folin and Denis (1912) for blood; Edwards and Condorelli, method of Condorelli (1922).

jection confirm those previously reported by Marshall and Grafflin, Grollman and Smith. These experiments demonstrate the rather striking fact that, after injection, the urinary concentration of urea is consistently significantly higher than the plasma level. One experiment is given in detail in the protocol. In two similar experiments, at lower plasma levels, the results were as follows: (*A*) plasma urea 24.7, urine urea 30.9 mgm. per cent, U/P ratio 1.25; (*B*) plasma urea 53.1, urine urea 63.0 mgm. per cent, U/P ratio 1.19. Whether the significantly higher concentration of urea in the urine represents a fundamental characteristic of the tubular membrane in this species cannot be stated positively at the present time. Although there is as yet no evidence for the reabsorption of water by the aglomerular tubule, the possibility must be considered; and there is furthermore the as yet untested possibility that water may be reabsorbed from the urine somewhere along the drainage system of the kidney (collecting ducts, ureter, bladder).

Goosefish; weight 12 kg.

		Urea in mgm. per cent		U/P ratio
		Plasma	Urine	
9:00 A.M.	Blood 1; urine 1. 40 cc. 50% urea intramuscularly	0.2	0.3	
10:10	Bladder emptied			
10:44	Blood 2	71.0		
11:12	Urine 2		95.0	
3:00 P.M.	Bladder emptied			
3:25	Blood 3	114.0		
3:51	Urine 3, 9.0 cc.		145.0	1.27
4:15	Blood 4	113.5		
4:42	Urine 4, 7.0 cc.		143.0	1.26

SUMMARY

Data upon plasma and urinary urea at varying rates of urine flow are presented for the sculpin and flounder. In addition, a few figures are reported for the daddy sculpin, sea raven, and wrymouth. From a comparison of the present data with those of Clarke (1934), glomerular filtration alone seems adequate to account for all of the urea excreted by the sculpin. Furthermore, urea may apparently be reabsorbed by the renal tubules in considerable amounts, particularly in the lower, more normal range of urine flow. In the aglomerular goosefish, in the plasma and urine of which urea is normally present in only minimal amounts, injected urea appears in the urine in significantly higher concentration than is present in the plasma.

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BIOLOGICAL OBSERVATIONS ON THE MARINE FUNGI OF WOODS HOLE WATERS

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INTRODUCTION

While the rôle played by marine bacteria in the decomposition of organic complexes is appreciated by all interested in a study of the ocean, the activities of fungi in this respect are practically unknown. Indeed, it may be justly said that the very existence of fungi which complete their whole life history in the sea is not known to most students of marine biology.

Occasional references to these rare and peculiar organisms may be found in the European literature from the 1860's onward. In the accounts of the early students of the seaweeds certain structures generally supposed by them to be the reproductive organs of the algæ were described and figured, which in many cases have been found to be, in reality, extraneous, parasitic fungi. In fact, practically all of the few marine fungi thus far found have been described as parasites or saprophytes of various marine algæ.

Representatives of four groups of the fungi have been discovered in marine waters, namely, the Myxomycetes (Labyrinthuleæ), Phycomycetes, Ascomycetes, and Fungi Imperfecti. Of the alga-inhabiting species which are truly parasitic or hemi-parasitic, those of the Phycomycetes seem to predominate. Many of these are not only able to initiate the infection of a healthy host, but once the alga has been killed have the capacity to live saprophytically on the dead contents, often in company with bacteria and Protozoa which have entered as secondary agents of destruction.

Only two papers have thus far appeared which deal at all extensively with marine fungi (Petersen, 1905; Sparrow, 1934) and both of these are concerned with forms collected in northern Europe. Twenty-three different organisms are described in these papers, all but one of which (*Pythium marinum* Sparrow) are ordinarily included in the order Chytridiales of the Phycomycetes. In our own waters two undoubted Phycomycetes have thus far been reported from the Pacific coast

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[1] Contribution No. 99.

(Zeller, 1918), while the Atlantic side has apparently yielded but a single one (Martin, 1922). Recently, Papenfuss (1935) has mentioned a chytridiaceous parasite of the brown alga *Ectocarpus siliculosus* Dillw. which he states attacked about 50 per cent of the unilocular sporangia, but he does not name the organism. This material was collected in the vicinity of Woods Hole, Mass.

The present investigation was initiated with two points in mind: first, to determine whether or not marine Phycomycetes were actually present in Atlantic coastal waters—a point not altogether evident from the extant literature—and secondly, to determine, if such were found, the nature and extent of their activities in the cycle of decomposition of marine organic complexes. This paper embodies the results of the work done mainly during the summer of 1934, and, to a lesser extent, a portion of the summer of 1935 on the first of these objectives and includes not only an enumeration of the fungi found in the vicinity of Woods Hole, Mass., but in addition, pertinent notes on their morphology, life history, and taxonomy.

Fungi Collected

1. *Labyrinthula sp.* Saprophytic in cells of *Rhizosolenia spp.*, bottom of Great Harbor, August 14, 1934.

In debris dredged from the bottom of Great Harbor during a heavy " flowering period " of diatoms, large numbers of *Rhizosolenia spp.* were found in various stages of decomposition. In these cells there were often found the motile stage of a *Labyrinthula* (Text Fig. 35), sometimes in company with a Protomyxa-like protozoan, or a small chytrid. The fusiform swarm cells of the *Labyrinthula*, which were 12 μ long by 5 μ in diameter, made their way in and upon the algal cells by gliding along their tenuous pseudopodia. The most anterior of these cells, which was not preceded by any protoplasmic attenuation, displayed remarkable powers of amœboid movement. No solid particles could be detected within the swarm cells.

The uncertain taxonomic status of the species of this peculiar group does not allow of any specific designation of the present form. Morphologically it does not seem very different from *Labyrinthula macrocystis* described from marine algæ by Cienkowsky (1867), from the common inhabitant of diseased *Zostera* (Renn, 1934), nor, indeed, from certain fresh-water forms.

Very frequently in the debris which collects in the bottom of aquaria containing marine algæ, species of *Labyrinthula* are observed, and it is probable that they occur much more frequently than records in the literature indicate—a fact also evident from Miss Jepps's (1931) paper.

No cases of actual parasitism of algæ by these forms were observed, and at Woods Hole, at least, the organism on *Rhizosolenium* appears to lead a saprophytic existence. Experiments to test the pathogenicity of various strains of *Labyrinthula* are greatly needed.

2. ?*Labyrinthula Chattoni* Dangeard. Parasitic(?) in *Cladophora sp.*, vicinity of Woods Hole, throughout the summer.

Very commonly in material of *Cladophora* which has been maintained in the laboratory for some time there will be found occasional greyish cells within which are rosettes of highly refractive material, generally surrounding a central mass of compacted brown granules—the remains of the host chloroplasts. These rosettes are divided radially into a varying number of segments from which at maturity numerous very minute amœboid bodies emerge. According to Dangeard (1932), these are the motile spores of the fungus, and he has figured numbers of spindle-shaped bodies which unite to form a net-plasmodium. In the present material no fusiform shape was ever assumed by the spores, although the bodies from which they emerged—considered by Dangeard to be sori—resembled closely those of his species; neither was a net-plasmodium formed, and for these reasons the writer considers a further study of the Woods Hole fungus necessary before definitely naming it.

3. *Protomyxa*-like parasite of *Bryopsis*. Parasitic in *Bryopsis plumosa,* vicinity of Woods Hole, summer of 1934, June, 1935; New Bedford Harbor, Mass., July 24, 1934.

In the course of studies made on a fungous parasite of *Bryopsis* (*Sirolpidium*) there was frequently found an exceedingly virulent parasite which is undoubtedly a protozoan. However, since the animal occurred in such abundance and very often in competition with the fungus, both usually consuming the entire contents of infected cells, it would seem of interest to include it here.

Infected cells were readily noted by their blackened appearance, a pathological symptom also produced by the fungus. The method of entrance of the parasite and the nature of the unit accomplishing this were not determined. Once established, the organism gradually enveloped part or all of the algal content which soon became discolored within its body. The shape of the invader was variable but in general resembled a giant amœba from any part of the surface of which delicate, often strongly contractile threads emerged. Very marked movement of these threads could be detected and frequently broadly fusiform or spherical enlargements containing algal material could be seen moving along them

(Plate I, Fig. 6) in much the same "trolly-wire" fashion as that observed in *Labyrinthula.*

In exhausted algal cells the animal seemed to go into a resting stage and to surround itself with a slightly thickened, rigid pellicle. The engulfed host contents were by now nearly black, and it was these cylindrical bodies with rounded ends which were so evident macroscopically.

A similar organism has been found very frequently in decaying *Rhizosolenia spp.* dredged from the bottom of Great Harbor and in dead cells of *Ectocarpus* from Gay Head.

4. *Ectrogella perforans* H. E. Petersen. Parasitic in *Licmophora abbreviata*, summer of 1934, June, 1935; *Striatella unipunctata*, summer of 1934, June, 1935; vicinity of Woods Hole; New Bedford harbor, July 24, 1934; Gay Head, Martha's Vineyard, Mass., July 16, 1934.

This virulent parasite is the commonest marine fungus in the vicinity of Woods Hole and during the summer it is almost impossible to find stands of *Licmophora* and *Striatella* not heavily infected with it. It not only occurs in all the littoral regions in the immediate vicinity of Woods Hole but has also been found in equal abundance in New Bedford Harbor and Gay Head. It is probable that it will be found along our coasts wherever the host plants grow. During the month of July in particular the fungus may occur in epidemic proportions (Plate III, Fig. 2). As many as 88 per cent of the diatoms (*Licmophora*) in a single mount containing many hundreds have been observed to be infected.

Since the present form presents no significant differences in morphological features from that recently described by the writer (1934) from Danish material found in the littoral of the Kattegat, it will not be dealt with in detail here, save for certain hitherto unknown phases.

In both *Licmophora* and *Striatella* there was ordinarily present in one infected diatom only a single, spherical, highly refractive thallus, although occasionally 2–5 were observed. This varied from 25–35 μ in diameter and lay loosely in the center of the host cell.

A good opportunity was afforded in several instances to determine the method of entrance of the parasite into the host cell, a feature not previously observed. Heavily infected colonies of diatoms contained many cells, to the outer surfaces of which adhered numerous minute (3 μ in diameter) quiescent cystospores of the fungus. The small size of these bodies made observations difficult, but in several cases spores were found from each of which a tenuous, needle-like penetration tube had emerged and had pierced the host wall. It is probable that, like other parasitic chytrids, the contents of the spore then flowed through the tube into the algal cell, leaving the wall of the cyst outside. Such empty cystospores were found, although the actual passage of material into the cell was not witnessed. Once inside, the refractive fungous protoplasm soon assumed a central position in the host cell, possibly attaching itself to the nucleus of the diatom. Growth into the mature, somewhat spherical sporangium then ensued. The star-like arrangement of the chloroplasts of the host was soon destroyed and, as the fungus developed, their individual structure was broken down and their normal color lost. At no time was any host material observed to be ingested directly nor did the fungus exhibit any signs of amœboid motion. As the *Ectrogella* increased in size, the disintegrating host material usually became collected around the periphery of the parasite, where it gradually became reduced to a small amount of granular, chestnut-brown residue. The nature of this unassimilated material is not known.

There then took place a transformation of the contents of the thallus into innumerable, minute zoöspores. These were discharged through 1–5 stout tubes into the water. A point of great morphological and taxonomic interest was determined from the spores of this material. Petersen (1905) states that in this species they are anteriorly uniciliate, the cilium being directed posteriorly when the spore is in motion. In the Danish material previously observed, the writer (1934) was unable to determine the ciliation, but at Woods Hole, the spores were found to be biciliate. This finding was confirmed in many instances. The body of the zoöspore is pyriform, somewhat curved, and at the narrower, forwardly directed end there is usually a refractive droplet. The two, perhaps anteriorly attached, cilia are directed oppositely when the spore is in motion. The relative lengths of these two structures could not be determined. Motion through the water was of an uneven, twisting character. The resemblance of these spores to those of *Sirolpidium* (Sparrow, 1934) is very marked.

From these observations, the parasite can now with real reason be placed in the genus *Ectrogella* as amended by Scherffel (1925).

At no time during the process of disintegration of the host cell could the presence of any other type of organism be detected. Bacteriological stains were employed to determine whether or not bacteria assisted in the process of disintegration, but none was ever observed. Indeed, the fungus had apparently so completely absorbed the available nutriment that even after infected cells had become broken or had fallen apart, bacteria and Protozoa were seldom seen feeding on the residual material.

It was very evident that not only could the fungus initiate the infection, but once inside, could bring about, unaided by other agencies, the almost complete disintegration of the contents. Further, the thallus, derived from a single zoöspore, at maturity became transformed into a myriad of swimming spores, each of which was potentially able to infect another diatom. Hence, in a region with an abundance of favorable host material, the rapid and widespread dissemination of the parasite could be quickly accomplished.[2] Indeed, it is remarkable in view of the high percentage of infection observed, that the two species of diatoms were able to maintain themselves at all. It is hoped that a more comprehensive study of this disease can be made in the future.

5. *Eurychasmidium tumefaciens* (Magnus) n. gen. Parasitic in the nodal cells of *Ceramium diaphanum,* Sheep Pen Cove, Woods Hole, August 27, 1934, collected by C. Jao; Little Harbor, Woods Hole, August 29, 1934, collected by the author.

Eurychasmidium n. gen. Plasmodial thallus entirely intramatrical, one-celled, filling the distended host cell, forming at maturity many discharge tubes which pierce the host wall; zoöspores biciliate, encysting near the sporangium after discharge, ultimately emerging as motile zoöspores. Resting spores not observed.

Thallus plasmodialis, omnino intramatricalis, unicellulatus, cellulam matricis distentam implens, maturitate formans multa ostiolata perforantia parietem cellulæ matricis; zoosporæ biciliatæ, encystantes extra sporangium post emissionem, e cystis emergents velut sporæ motiles. Sporæ perdurantes non observatæ.

Eurychasmidium tumefaciens (Magnus) comb. nov.
Syn. *Chytridium tumefaciens* Magnus.
Olpidium tumefaciens (Magnus) Berl. et de Toni.

Thallus filling the host cell; spherical or irregular and lobed; producing a marked enlargement of the host cell and stimulating the host to form clusters of lateral branches; becoming transformed into a single sporangium, the spherical ones 100–110 μ in diameter, the lobed examples up to 110 × 200 μ long. Zoöspores formed within the sporangium (ciliated?), upon discharge through as many as 30 discharge tubes forming a dense motionless cluster near the sporangium; encysting, the somewhat loosely disposed cysts being angular and 4 μ in greatest width; emerging from the cysts after a period of quiescence as ellipsoid, biciliated spores, 3 μ in diameter by 5 μ in length. Resting spores not observed.

This remarkable fungus which usually attacked only the nodal cells of the *Ceramium* possesses many points of interest. In its method of infection, developmental features, and effect on the host cell it resembles to a marked degree *Eurychasma Dicksonii* (Sparrow, 1934). However, it differs from this genus since it always remains intramatrical, since the zoöspores undergo a period of encystment outside the sporangium, not inside, as in *Eurychasma,* and since it produces adventitious lateral branches and a more marked swelling of the host cell (Plate I, Fig. 1).

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[2] One curious evidence of the virulence of this fungus was noted. In a mount containing many swimming spores, a species of *Vorticella* was observed which had captured 10 or more zoöspores. The latter instead of being digested by the animal, had reversed the process and had absorbed the contents of the protozoan and were forming sporangia!

The earliest stages observed in the development (Text Figs. 14, 15) showed an already well-established thallus easily distinguishable in the distended host cell by the presence of numerous irregular, refractive bodies. As growth proceeded, the disintegrated chloroplast material became more granular while the host protoplasm assumed a vacuolate character (Text Fig. 18). Coincident with the increase in size of the fungus there was a strong distention of the host cell (Text Fig. 17).

Infection is unquestionably initiated by the zoöspore, probably in the same manner as described for *Ectrogella perforans.* While actual observations on this point were lacking, there were occasionally observed instances similar to Text Fig. 16, in which the empty cyst and remains of the penetration tube were still present on the infected cell. The sequence of changes ensuing in the fungous protoplasm itself as growth proceeded were entirely similar to those described by Petersen (1905) for *Eurychasma,* including the characteristic " stade écumeux " (Text Fig. 19).

During enlargement of the host cell, a pronounced thickening of the algal wall material was noted. Also, certain adjacent nodal cells were stimulated to divide and there was produced in the vicinity of an infected cell a number of curved, stunted, lateral branches which gave a " bushy " appearance to the region (Plate I, Fig. 1). Such places could easily be detected with a hand-lens. Since the fungus was not observed to form any rhizoidal system, but was confined to a single cell, it would seem that some sort of substance was produced in infected cells which diffused to adjacent ones and stimulated them to abnormal growth.

Occasionally, infected apical cells of *Ceramium* were found with 2–6 thalli in them, probably the result of multiple infection. If these were produced by the same fungus as found in the nodal cells, it is interesting to note that here, very little hypertrophy occurred.

Unfortunately, the final stages in the formation of the zoöspores and their discharge from the sporangium through many discharge tubes were not observed, in most cases evacuation of the spores having taken place before the preparations were made. Nor could means be found to promote maturation and discharge of sporangia under observation. Hence, it is not known whether the spores emerge by ciliary action or are forced out of the sporangium. Once emerged, however, they form a dense cloud around the infected filament, each spore being surrounded by an angular wall (Text Fig. 21). These cystospores are not connected with one another as they appear to be in the sporangium of *Eurychasma* but are loosely disposed in the medium (Text Fig. 20). After an unknown period of quiescence the protoplasm of each of these cysts emerges, probably through a pore formed in the wall, and undergoes near the empty shell a rocking and fashioning which terminates with the production of the fully formed spore. The body of the spore is somewhat elliptical, generally possessing a single, minute refractive droplet, and two cilia—the latter point being determined from a study of killed and stained material. Further observations on the orientation, length, and points of attachment of these cilia are needed.

This fungus is a true parasite, initiating and carrying to completion the destruction of the host cell unaided by other agencies. Unlike certain other marine fungi, however, it does not seem able to continue its destruction of the alga after the latter has been noticeably weakened, as, for example, by remaining under laboratory conditions for a time. The remainder of the host plant, save in the immediate vicinity of an infected cell appears unaffected by the incursion of the fungus.

6. *?Petersenia sp.* Saprophytic in *Ceramium diaphanum*, Little Harbor, Woods Hole, August 29, 1934.

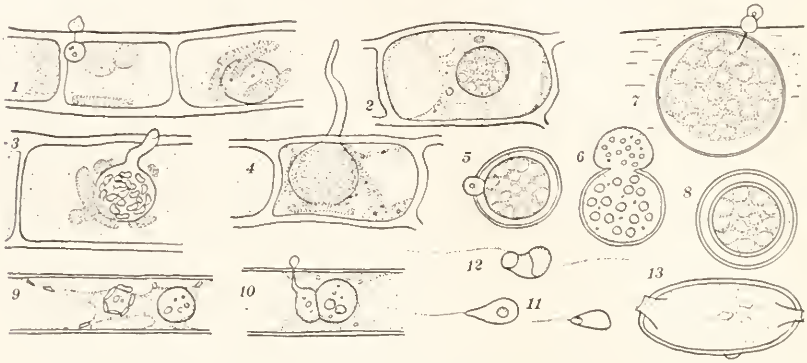
In material of *Ceramium diaphanum* which had been kept in the laboratory in an aquarium for several weeks, there was found a " Pleotrachelus "-like fungus, probably to be regarded as a saprophyte in the alga. In contrast to the previously described fungus on this alga, the present form was found only in the internodal cells and in further distinction was never observed to produce any hypertrophy of the host.

One to many thalli of varying size (20–96 μ in diameter) were formed in a single internode (Plate II, Fig. 4). While maintaining in general a spherical shape, these thalli occasionally assumed irregular forms, though no lobed examples were found.

The mature sporangium was characterized by its relatively large size, attaining frequently a diameter of 96 μ or more, although occasional specimens measured only 20–25 μ. At maturity up to ten or more narrow, distally tapering, radiating discharge tubes (about 7 μ in diameter) were formed (Plate II, Fig. 3) which bored through the cell wall of the alga. Zoöspores and resting spores were not observed, and hence the exact generic disposition of the fungus awaits the results of further investigations. If, as is probable, the swarmers are found to be biciliate, affinity with *Olpidiopsis* and more especially *Petersenia* would be indicated.

7. *?Petersenia sp.* Parasitic in eggs of a microscopic animal (rotifer?), Sheep Pen Cove, Woods Hole, August, 27, 1934.

Adherent to filaments of *Ceramium diaphanum* there was an abundance of eggs of a microscopic animal, probably belonging to the *Rotiferæ,* members of which were swarming in the mount. Some of these were parasitized by a fungus allied to *Petersenia* and *Olpidiopsis.* The thallus occupied the whole of the interior of the egg and at maturity was transformed into a single sporangium 50 μ long by 20 μ in diameter. This was provided with 1–3 broad (8 μ in diameter), short, discharge tubes (Text Fig. 13). The actual formation and discharge of the zoöspores was not witnessed, although a number of these bodies which had failed to emerge were observed within the sporangia. These were reniform, of the "laterally biciliate" type, 4 μ long by 2 μ in diameter, and were very similar to those formed by *Pythium.*



TEXT FIGS. 1–13. Figs. 1–8, *Petersenia Andréei;* Fig. 1, early stage in the formation of the thallus in *Ectocarpus,* the collapsed cyst of the infecting zoöspore and the penetration tube still present, a more mature thallus is seen in the right-hand cell, × 376; Fig. 2, more mature thallus containing characteristic refractive bodies, × 376; Fig. 3, mature, unopened sporangium showing some of the zoöspores which are actively moving within it, × 376; Fig. 4, mature sporangium with long extramatrical discharge tube, × 376; Fig. 5, mature resting spore with male cyst adherent to it, × 600; Fig. 6, conjugation of thalli previous to resting spore formation, × 525; Fig. 7, immature resting spore with two cystospores, which have apparently conjugated, attached by a penetration tube to it, × 760; Fig. 8, mature resting spore showing interpretation of wall structure, × 600. Figs. 9–11, (?)*Olpidium sphacellarum;* Fig. 9, young, spherical thallus in hair of *Sphacelaria,* × 525; Fig. 10, young thallus attached to nucleus of host cell, the cyst of the infecting zoöspore and penetration tube still persistent, × 525; Fig. 11, interpretation of two zoöspores, free-hand. Fig. 12, *Petersenia Andréei,* interpretation of zoöspore, free-hand. Fig. 13, *Petersenia sp.* in egg of rotifer, discharged sporangium containing a few zoöspores, × 376.

No bacterial or other biological agencies appeared to assist in the destruction of the eggs. It is hoped that more material of this fungus can be found in order that a further study of its method of infection, as well as the formation and discharge of the zoöspores can be made. From the ciliation of the spores there seems little question of its relationship to *Petersenia*.

8. *Petersenia lobata* (Petersen) Sparrow. Parasitic on *Callithamnion roseum*, growing on *Chorda filum*, dredged from between East Chop and West Chop Lighthouses, Vineyard Haven, August 22, 1934; Sheep Pen Cove, Woods Hole, August 23, 1934.

This peculiar fungus was abundant in certain tufts of the host, but it was never observed to attack all the plants on a single *Chorda*. Cells near the base of the tuft were most frequently infected.

The predominating type of thallus found in this material was cylindrical with rounded ends, although there occurred numerous lobed examples, often of very bizarre appearance (Plate II, Figs. 1, 2).

A morphological and taxonomic discussion of this fungus has recently been given by the writer (1934) and since no unusual features were displayed by the American material, it will not be repeated here.

In the early stages of invasion of the host cell, the parasite is unassisted by other organisms. However, in old infections, an abundance of bacteria and Protozoa are always found and it seems probable that the fungus initiates the destruction of the host cells, being aided in later stages by these other agencies.

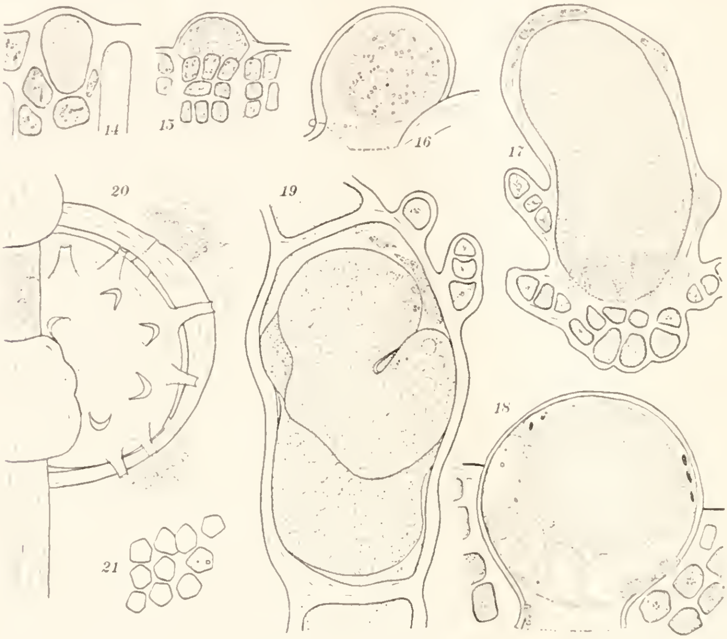
9. *Petersenia* (*Olpidiopsis*) *Andréei* (Lagerheim) comb. nov.
Syn. *Pleotrachelus Andréei* Lagerheim, 1899.
Pleotrachelus ectocarpi Jokl, 1916.

?Parasitic in *Ectocarpus siliculosus*, dredged off Gay Head, Vineyard Sound, July 16, 1934; Great Harbor, Woods Hole, August 24, 1934.

Frequent examples of extensive infection of host plants were found in the material from Gay Head, and quite commonly large fronds were observed with nearly every cell showing evidence of fungous activity. Such plants were easily recognized by their slightly greener appearance. So extensive was the infection that it could not be determined whether the fungus was the primary cause of the destruction of the alga, or whether it was only a saprophyte.

There was little doubt as to the method of entrance of the fungus. Numerous examples similar to Text Fig. 1 were observed which clearly showed that the zoöspore, after coming to rest on the surface of the host, had gained entrance by the formation of a penetration tube through which the content of the spore passed into the interior. It was the disorganized condition of the host cell contents even in early stages of infection which seemed to indicate that the fungus was probably only weakly parasitic or even saprophytic.

Further development involved the enlargement of the thallus (Text Fig. 2), accompanied by a gradual disintegration of the host contents. One to three thalli were usually formed in a single cell. The mature sporangia lay loosely in the host cell and were generally spherical and 18–20 μ in diameter (Text Fig. 4). A single discharge tube, 4–5 μ in diameter, was formed which often extended for as much as 78 μ out into the water. Occasionally, the tube did not achieve a correct orientation, in which case it passed through a number of host cells before reaching the outside.



TEXT FIGS. 14–21. *Eurychasmidium tumefaciens.* Figs. 14, 15, early stages in the formation of the thallus in *Ceramium diaphanum*, × 225; Fig. 16, more mature thallus showing at the bottom of the left-hand side the remains of the infecting zoöspore and its penetration tube, × 376; Figs. 17–19, strongly hypertrophied host cells with vacuolate thalli of the fungus in them, Fig. 19 shows one of the infrequently formed lobed thalli, all × 225; Fig. 20, empty sporangium showing the many discharged tubes formed and a few of the encysted zoöspores, the latter formed a dense cloud around the sporangium, × 225; Fig. 21, cysts formed by the discharged zoospores, from all but one of which the spore has emerged, × 760.

The zoöspores were fully formed within the sporangium by a process of progressive cleavage. After maturation, and before dissolution of the tip of the discharge tube, they underwent a prolonged period of violent individual movement and " milling around " (Text Fig. 3). Upon the opening of the discharge tube they escaped by their own efforts from the sporangium. In their morphology, these spores (Text Fig. 12) are strikingly like those of *Pontisma* (Sparrow, 1934), being irregularly piriform, arched, 5 μ long by 3 μ in diameter, and possessing at the narrow, forward end, as well as at the broader posterior one, a strongly refractive region. As has been suggested for *Pontisma,* a broad central, transverse groove and a " doubling-over " of the ends may account for this peculiar appearance. However, because of their small size, observations on moving spores are difficult. From the more anterior part of the groove there arise two cilia which appear to be of nearly equal length and are disposed in opposite directions along the long axis of the spore. These observations confirm Petersen's (1905) brief statement that the spores are laterally biciliate.

The spores moved in an erratic manner through the water. In hanging drop cells many of them germinated, each giving rise to a single, slender, very irregular tube, often of considerable length (30 μ).

Resting spores occurred in abundance (Plate I, Fig. 8). It will be recalled that in the Danish material of what is probably the same species, the writer (1934) showed that resting spore formation was apparently preceded by a conjugation between thalli of unequal size, the contents of the smaller passing into the larger which became the resting body. In the present fungus a similar phenomenon was observed in many instances and there seems little question that the resting spores are produced as a result of a sexual act almost identical with that found in the genus *Olpidiopsis.* Communication between the two cells was affected by the formation of a pore, no evidences of a definite fertilization tube ever being found. While, in general, the two conjugating thalli were more nearly equal (12 μ, 23 μ) (Text Fig. 6) than was observed in the Danish material (17 μ, 5 μ), occasionally they differed greatly in size (20 μ, 8 μ) (Text Fig. 5).

The mature resting spore was spherical, 20–23 μ in diameter, and appeared to be surrounded by a thick wall. However, under high magnification it was seen that this apparent wall could be resolved into the following structures: an outer, very definitely thickened membrane surrounding the whole, an inner membrane of the same thickness surrounding the refractive contents, and between them, a clear zone (Text Fig. 8). Whether the spore itself is to be regarded as relatively thin-walled and resting within a larger structure, or whether all of these parts are fused into a common surrounding layer, is not clear. Further observations on the development of the resting spore will be necessary to clarify this point.

Another puzzling feature was the absence in some cases of companion cells on the mature resting spores. Either the cyst was hidden from view by the body of the spore, or it soon disintegrated, or conjugation occurred in some other manner. Some evidence in favor of the latter case is given by the condition shown in Text Fig. 7. Here, attached to an immature resting spore by a tenuous penetration tube, are the cysts of two zoöspores which apparently have conjugated. It is entirely possible that after this process the merged plasma entered the host and there formed the resting spore.

In material of *Ectocarpus* in a floating *Chorda* complex collected in Great Harbor, Woods Hole, what appeared to be germinated resting spores were found. In the process of germination, the inner wall had apparently been absorbed, a long evacuation tube formed, and the contents, undoubtedly in the form of zoöspores, had been discharged through it.

EXPLANATION OF PLATE I

All photographs were made from living material with the exception of Plate I, Figs. 3, 5, and Plate II, Figs. 4, 5, which were made from material mounted in glycerine and stained with eosine. The approximate magnifications are given in each instance.

Fig. 1. *Eurychasmidium tumefaciens*, parasitic in *Ceramium diaphanum*. Two hypertrophied cells are shown; the right-hand filament has produced lateral branches in response to the incursion of the fungus. The indistinctness of the right-hand figure is due to the presence of a cloud of spores which has just been discharged from the sporangium. × 80.

Fig. 2. *Thraustochytrium proliferum*, saprophytic on *Bryopsis plumosa*. The right-hand figure is a sporangium with the spores about to be discharged; the highly refractive body at the base of the sporangium is the fundament of the new sporangium which will develop after discharge of the spores. The left-hand figure may possibly be an immature resting spore. × 380.

Fig. 3. (?) *Olpidium sphacellarum*, parasitic in *Sphacelaria cirrhosa*. An empty sporangium with two lateral, oppositely placed discharge tubes. × 330.

Fig. 4. *Sirolpidium bryopsidis*, in *Bryopsis plumosa*. Typical olpidioid thalli formed from the fragmentation of an originally filamentous thallus. × 380.

Fig. 5. *Pontisma lagenidioides*, saprophytic in *Ceramium diaphanum*. Habit of the segmented thallus in the alga. × 380.

Fig. 6. *Protomyxa*-like animal parasitic in *Bryopsis*. The delicate pseudopodium of the animal bearing a large spherical and two small, fusiform enlargements may be seen extending from the top to the bottom of the photograph. × 380.

Fig. 7. *Sirolpidium bryopsidis* in *Bryopsis*. Thalli after 24 hours exposure to dripping sea-water. Compare with Fig. 4, which is under completely submerged conditions. × 380.

Fig. 8. *Petersenia Andréei*, parasitic (?) in *Ectocarpus*. Resting spores in cells of filament. × 250.

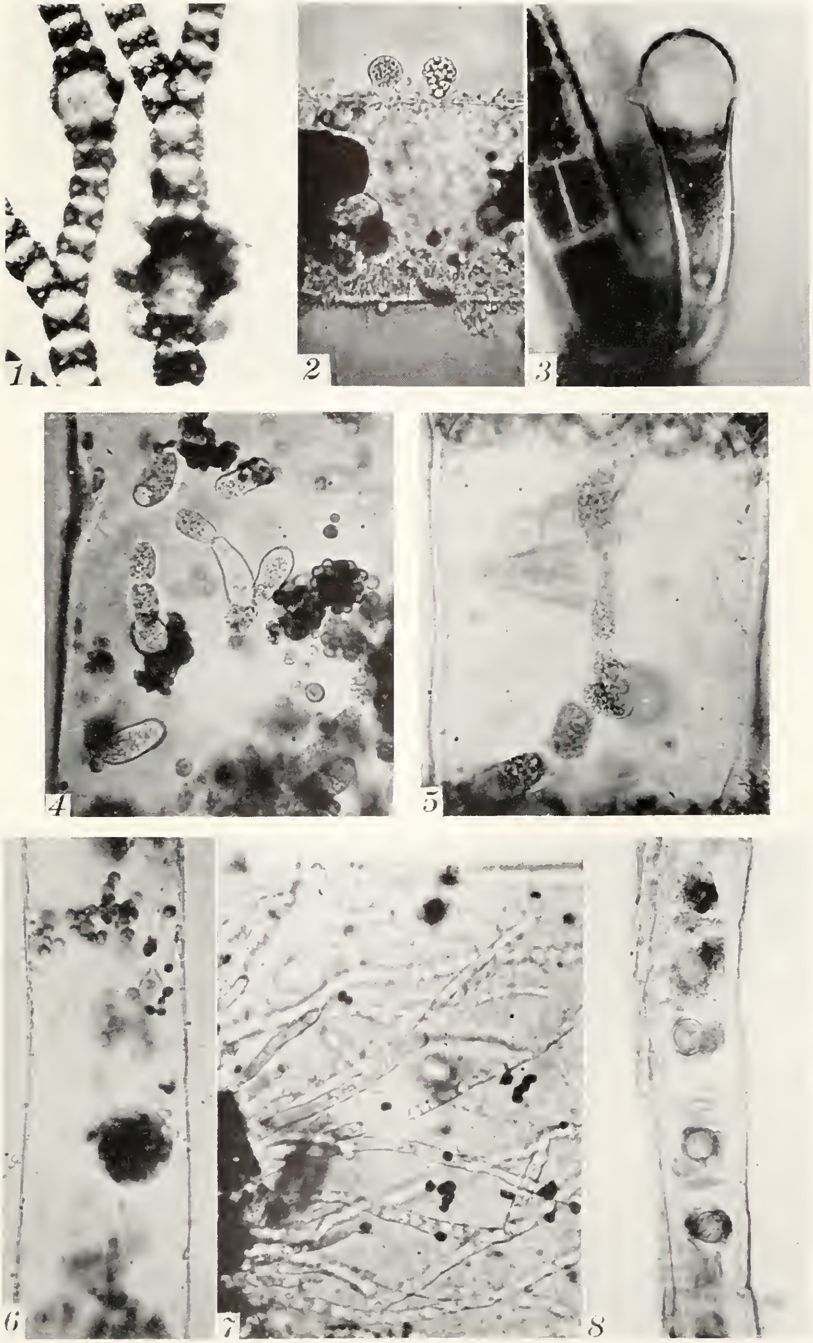


PLATE I

While a *Protomyxa*-like organism was abundant in certain cells of the *Ectocarpus*, the parts of the host occupied by the fungus were free from the animal, and, so far as could be determined, from other biological agents of disintegration as well.

10. (?)*Olpidium sphacellarum* Kny. Parasitic on *Sphacelaria cirrhosa*, Pine Island, Woods Hole, June 24, 1934.

This fungus was found in only one collection of the alga, although a search was made for it throughout the time the host was available. It attacks chiefly the apical cells of the lateral branches (sporangia?) and the colorless setæ. The rest of the plant appears immune and is not visibly affected by the presence of the fungus.

Early stages in the development were found, particularly in the colorless setæ. The infecting unit is the zoöspore, which comes to rest on the host, encysts, and produces a tenuous tube which penetrates the algal wall and through which the contents of the spore are conducted into the cell. In many cases the young thallus attaches itself to the nucleus of the host cell (Text Fig. 10). It then gradually enlarges into a spherical (Text Fig. 9), or often cylindrical structure with rounded ends. No hypertrophy is evident. In infected lateral branches (sporangia?), however, a pronounced swelling occurs (Plate I, Fig. 3, Plate II, Fig. 5). The disintegration of the cell contents is almost complete and there is generally left only a mass of irregularly-shaped, colorless granules. The mature sporangia are nearly spherical and 35–45 μ long by 30–35 μ in diameter. One, or occasionally 2–3 short, stout tubes, 8–10 μ in diameter, are formed through which the mature zoöspores are discharged. Before these are ejected they undergo a period of active swarming, eventually being liberated very rapidly at the dissolution of the tip of the discharge tube. They are minute, being 4 μ long by about 2 μ in diameter, and have a somewhat piriform body (Text Fig. 11), near the broader end of which there is a prominent refractive globule. The position of this globule may change while the spore is motile. Com-

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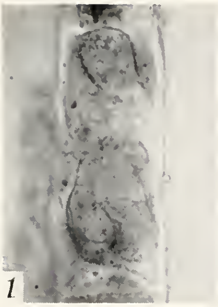
EXPLANATION OF PLATE II

Figs. 1, 2. *Petersenia lobata*, in *Callithamnion roseum*. Two thalli, showing the peculiar lobing sometimes encountered. × 260.

Fig. 3. *Petersenia sp.*, saprophytic in *Ceramium diaphanum*. An empty sporangium with three discharge tubes visible. × 260.

Fig. 4. Same fungus, showing the spherical, immature thalli and several discharged sporangia. × 260.

Fig. 5. (?)*Olpidium sphacellarum*, parasitic in *Sphacelaria*. Two thalli, one in the colorless hair, the other in the hypertrophied tip of the lateral branch. × 330.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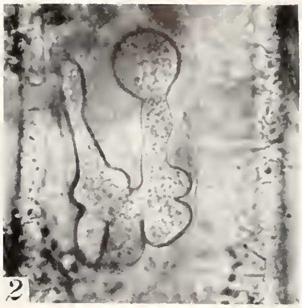
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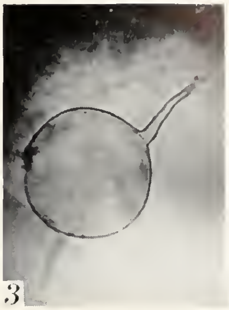
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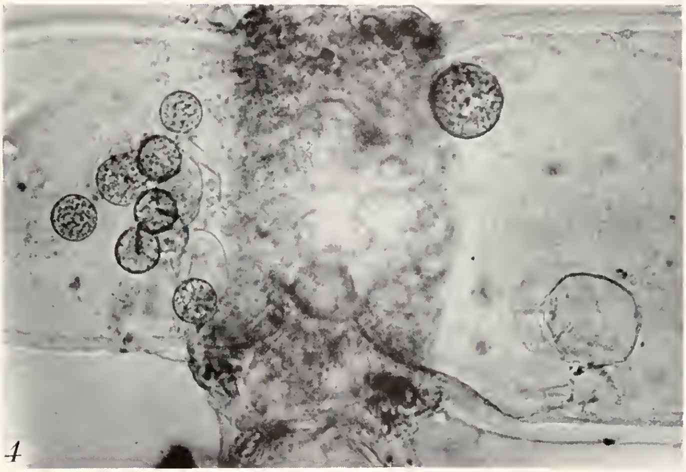
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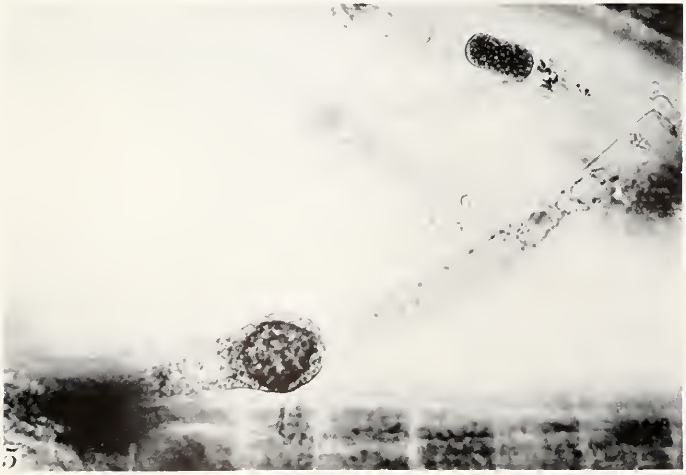
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PLATE II

plete data on the ciliation of these spores were not obtained. It is certain that they possessed a very prominent, forwardly directed cilium which appeared to remain nearly stationary during the period of motility. It is probable that another, rapidly vibrating, possibly shorter, posteriorly directed cilium was also present but escaped detection. The zoöspore exhibited a very peculiar type of movement which was characterized by periods of even, forward progression, alternating with sudden stops and change of direction. There appeared to be practically no rotation of the body during movement.

The fungus was a true parasite, invading only the healthy cells of the host and accomplishing alone the disintegration of the contents. With the decline of the alga under laboratory conditions no further fungous activity was noted.

While considered a species of *Olpidium*, it is evident from what is known of the ciliation of the spores that this fungus bears only a superficial resemblance to this genus. If, as is highly probable, the zoöspores are found to be biciliate, it should be placed near *Olpidiopsis* and *Petersenia*.

11. *Pontisma lagenidioides* Petersen. (?)Saprophytic in the internodal cells of *Ceramium diaphanum*, Little Harbor, Woods Hole, July 8, 1934.

Since details of the morphology of this species have been given in a recent paper (Sparrow, 1934), they will not be repeated here. No significant differences from the Danish material were apparent, except for the fact that the Woods Hole fungus (Plate I, Fig. 5) could at the most be called only weakly parasitic on the *Ceramium*. Indeed, only after the alga had been maintained in the laboratory for several weeks did the fungus appear in appreciable quantity. While occasional thalli were formed in apparently living cells free from other organisms, they occurred more commonly in dead and somewhat desiccated algæ along with bacteria and Protozoa.

12. *Sirolpidium bryopsidis* (de Bruyne) Petersen. Parasitic in *Bryopsis plumosa*, Woods Hole, throughout the summer, 1934; June–July, 1935; *Cladophora sp.* (saprophytic?), Woods Hole, July 1, 1935.

EXPLANATION OF PLATE III

FIG. 1. Plant of *Bryopsis plumosa* infected with *Sirolpidium bryopsidis*. The blackened areas caused by the fungus may be seen particularly well along the axes of the lateral branches. About natural size.

FIG. 2. *Ectrogella perforans*, parasitic in *Licmophora abbreviata*. A stand of the diatoms heavily infected with the fungus. Infected cells may be distinguished by the light, spherical sporangia of the *Ectrogella* within them. × 260.

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Plate III

As in the preceding case, the Woods Hole material of this fungus showed no morphological differences from that found in Danish waters (Sparrow, 1934) and this phase of the organism will not be dealt with again here. It might be noted, however, that the zoöspore effects penetration of the host cell exactly as in (?)*Olpidium sphacellarum.*

Macroscopically, heavily infected plants may be recognized by the presence of blackened areas along the fronds (Plate III, Fig. 1). The general aspect of the fungus in the alga is shown in the photomicrograph (Plate I, Fig. 4). While early in the course of the infection the fungus undoubtedly acted alone in the entering and the breaking down of the algal contents, in later stages it was often accompanied by swarms of bacteria and Protozoa. Mention has been made already (p. 238) of the peculiar Protomyxa-like animal which often competed with the fungus. Indeed, in some cases this association was so constant that had this animal not been entirely absent in the Danish material, it might easily have been supposed to be a phase in the life history of the *Sirolpidium.*

Since this fungus, by reason of the somewhat filamentous nature of its thallus, seemed the most suitable of these marine fungi for the purpose, attempts of a very preliminary nature were made to culture it. It had been previously noted that if infected *Bryopsis* plants were put in close contact with air, such as allowing water to drip over them rather than to submerge them entirely, the fungus underwent a remarkable transformation in body structure. Instead of the formation of typical olpidioid thalli (Plate I, Fig. 4), there were formed definitely filamentous, ramifying hyphæ (Plate I, Fig. 7). The resemblance of these to the hyphæ of the higher Phycomycetes was very striking.

Pieces of *Bryopsis* containing such hyphæ were placed in various nutrient solutions to determine whether or not further vegetative growth could be induced. Dextrose, mannose, galactose, and fructose were used in Seitz-filtered sea-water solutions of 0.5 per cent and 1.0 per cent and maintained at 19–20° C. Certain protein solutions were tried but fouled too rapidly from bacterial action to be of use. The amount of extramatrical growth was used as a criterion for the efficacy of the particular solution. Suitable controls in Seitz-filtered sea water alone were also set up. Since it was soon found that only the dextrose had a pronounced effect on growth, further solutions of this sugar were made up to determine its most favorable concentration. The results of these preliminary trials are given in Table I.

From these data it can be seen that the lower concentrations of sugar were most effective in stimulating extramatrical growth, whereas the higher concentrations, while they were not lethal to the fungus, seemed to have been definitely inhibitory. Attempts were then made to culture *Sirolpidium* on a solid medium by placing bits of the alga containing extramatrical growth on 0.5 per cent and 1.0 per cent dextrose solutions to which had been added either 0.5 per cent or 1.0 per cent agar. Further growth could not be induced. Studies on the cultivation of *Sirolpidium* are being continued with the hope that from the data obtained from this particularly favorable fungus a method can be devised for getting a number of these organisms into culture. If this can be accomplished, then specific information can be obtained regarding the nature of the activities carried on by these marine fungi.

TABLE I

Extramatrical growth of Sirolpidium in dextrose solutions at 19–20° C.

Solution per cent	Effect on growth			
	24 hours	48 hours	56 hours	80 hours
0.0 (control)	Practically none.	No change.	No change.	No change.
0.5	Excellent growth, up to 100 μ.	Up to 197.5 μ.	No change.	No change; mycelium fragmenting.
1.0	Good growth; up to 62–87 μ.	Up to 112 μ.	No change.	No change; mycelium fragmenting.
1.5	Very little; up to 20–30 μ.	Up to 100–137 μ.	Up to 140 μ.	Up to 175 μ.
2.0	None.	None.	Up to 20 μ.	No change.

13. *Chytridium polysiphoniæ* Cohn. Parasitic on *Polysiphonia fibrillosa,* Woods Hole, August 27, 1934; saprophytic on *Ceramium rubrum,* Woods Hole, August 24, 1934.

Details of the morphology and reproduction of this species are given in a previous paper (1934) by the writer. On *Polysiphonia* (Text Fig. 31), the Woods Hole fungus was an undoubted parasite, whereas it was only saprophytic on *Ceramium,* as was indicated by the whitened and disorganized contents of the alga. Bacteria and Protozoa were abundant in cells of the latter alga, but were absent in infected cells of the *Polysiphonia.*

14. *Chytridium megastomum* Sparrow. Parasitic on *Ceramium diaphanum,* Woods Hole, August 24, 1934.

No morphological differences from the Danish fungus (Sparrow, 1934) could be observed in this material. The sporangia occurred in great numbers on all parts of the alga; bacteria and Protozoa were, however, absent. An interesting feature of the parasitism of this fungus was the fact that the *Ceramium* appeared at the time of observation unaffected by the presence of the *Chytridium*. It seems probable, however, that even though the fungus itself did not exert a marked effect on its host, the innumerable punctures in the cell wall of the alga laid it open to entrance and attack by bacteria, and later, Protozoa. Such a sequence did occur when infected plants were maintained in a laboratory aquarium, but this does not necessarily indicate that such also takes place under natural conditions.

15. *Pleolpidium* (*Rozella*) *marinum* n. sp. Parasitic on *Chytridium polysiphoniæ,* Woods Hole, August 27, 1934.

In material of *Chytridium polysiphoniæ* parasitic on *Polysiphonia fibrillosa,* occasional sporangia were observed which were of larger diameter (30–45 μ) and possessed a thicker wall and more oleaginous contents than others. In several instances these were seen to discharge motile bodies through 1–3 pores (Text Fig. 33). The spores were minute, 3 μ long by 2 μ in diameter, ellipsoid, posteriorly uniciliate, and in contrast to those of *C. polysiphoniæ,* lacked a refractive oil droplet in their content. After a period of swarming, certain ones settled down on the surface of the sporangia of *C. polysiphoniæ* and were observed to produce a fine penetration tube (Text Fig. 32). Further development was not witnessed.

While this fungus might at first sight be considered a multiporous species of *Rhizophidium,* it is in reality a parasite of the *Chytridium,* closely allied to certain fresh-water species usually placed in the genus *Pleolpidium*. Reasons for referring all these species to Cornu's genus *Rozella* have been stated in a previous paper (Sparrow, 1936). Since no marine representatives of this genus have heretofore been described, the fungus is considered a new species, and is termed *Pleolpidium* (*Rozella*) *marinum* n. sp.

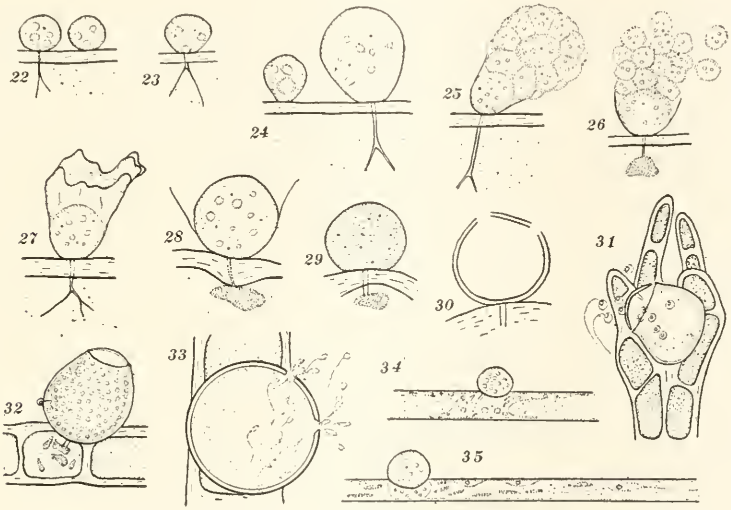
Pleolpidium marinum n. sp. Sporangium spherical, completely filling that of the enlarged host plant; 30–45 μ in diameter, at maturity forming 1–3 pores through which the zoöspores are discharged; zoöspores ellipsoidal, 3 μ long by 2 μ in diameter, posteriorly uniciliate, aguttulate. Resting spores not observed.

Parasitic on *Chytridium polysiphoniæ*.

P. marinum sp. nov. Zoösporangium globosum, cellulam matricis distentam omnino implens, 30–45 μ dia., maturitate 1–3 ostiolatis præditum per quæ zoösporæ emittuntur; zoösporæ ellipsoideæ, 3 μ long. v. 2 μ dia., posteriore uniciliatæ, aguttulatæ. Sporæ perdurante non observatæ.

16. *Rhizophidium discinctum* Petersen. Parasitic(?) on *Ceramium diaphanum,* Woods Hole, August 17, 1934.

A fungus probably identical with that found by Petersen (1905) on *Spongomorpha* and *Acrosiphonia* in Scandinavia was found attacking the nodal cells of the *Ceramium* which had been maintained in a laboratory aquarium for a week. It is probable that the fungus was only weakly, if at all, parasitic on the alga.



Text Figs. 22–35. Figs. 22–28, *Thraustochytrium proliferum,* on *Bryopsis plumosa,* all × 760. Figs. 22–24, various stages in the development of the sporangium; Fig. 25, mature sporangium showing basal body which will become the fundament of the secondary sporangium; Fig. 26, discharge of the zoöspores after the bursting and deliquescence of the upper part of the sporangial membrane; Fig. 27, discharged primary sporangium with secondary sporangium beginning to enlarge; Fig. 28, more mature secondary sporangium. Figs. 29–30, *Rhizophidium discinctum* on *Ceramium diaphanum;* Fig. 29, sub-spherical immature sporangium, × 376; Fig. 30, empty sporangium showing angular aspect and thick wall, × 376. Figs. 31–32, *Chytridium polysiphoniæ;* Fig. 31, discharging sporangium on *Polysiphonia,* × 376; Fig. 32, mature sporangium attacked by a zoöspore of *Pleolpidium* (Rozella) *marinum* × 376. Fig. 33, *Pleolpidium marinum,* Fig. 33, discharging sporangium of the fungus in *Chytridium polysiphoniæ,* × 376. Figs. 34–35, *Rhizophidium globosum*(?) on *Rhizosolenia sp.;* Fig. 34, sporangium with peg-like intramatrical tube, × 376; Fig. 35, sporangium apparently attached to nucleus of host, swarm spores of *Labyrinthula sp.* also attacking diatom, × 376.

The sporangia are at first spherical or hemispherical (Text Fig. 29), but at maturity, because of the formation of one or two laterally-placed discharge papillæ, become more angular. In some cases the upper part of the sporangium may be very definitely flattened. A further characteristic is the thickness of the sporangium wall, a feature very evident in discharged sporangia (Text Fig. 30). Because of the density of the host contents and especially because of the thickness of the algal wall, observations on the nature of the intramatrical system were difficult. So far as could be determined, this structure appeared to consist of a rather stout, unbranched tube. However, it is possible that it branched deeper in the host. Because of this uncertainty it is probably better to keep the fungus in the genus *Rhizophidium* than to transfer it to *Phlyctidium*, where, because of the unbranched rhizoid, it might be placed.

Martin's fungus (Martin, 1922), termed by him *Rhizophidium polysiphoniæ* (Cohn) Petersen, is probably referable to this species.

17. *Rhizophidium globosum* (Br.) Schröt.(?). Parasitic on *Bryopsis plumosa*, Woods Hole, August 8, 1934; saprophytic on *Rhizosolenia spp.*, Woods Hole, August 16, 1934.

The sporangia of the form on *Bryopsis* were spherical, 13–18 μ in diameter, and were attached to the host cell by a delicate, scarcely perceptible, branched, intramatrical rhizoidal system. In contrast to *Rhizophidium discinctum*, the even contour of the sporangium was maintained at maturity.

The fungus attacked only healthy cells of the *Bryopsis*. While the parasite at the time of observation did not appear to have any noticeable effect on the host, it undoubtedly paved the way for the more destructive activities of later invaders.

What may possibly be a closely related form was found in some abundance on cells of *Rhizosolenia spp.* dredged from the bottom of Great Harbor during a heavy "flowering period" of diatoms. The sporangia (Text Figs. 34, 35) were globose to sub-globose, 9–12 μ in diameter, and were generally found singly, attached to the mid-region of the diatom. A single, delicate rhizoid penetrated the alga and possibly attacked the nucleus. Discharge of the zoöspores was not observed and hence, the possibility that we are dealing with a species of *Chytridium* cannot be overlooked.

The fungus on *Rhizosolenium* was found only on obviously disintegrating diatoms, generally in company with a species of *Labyrinthula* (Text Fig. 35) and a *Protomyxa*-like animal. Flagellates were also frequently observed in such cells. No bacteria could be demonstrated in diatoms having both fungus and *Labyrinthula*, or fungus alone, in them.

Both of these fungi are only tentatively referred to the morphologically similar species inhabiting fresh-water algæ.

18. *Thraustochytrium proliferum* n. gen. n. sp. Saprophytic on *Bryopsis plumosa*, July 30, 1934; *Ceramium diaphanum,* August 17, 1934; Woods Hole, Mass.

Thraustochytrium n. gen. Spore upon coming to rest on the host cell encysting and producing an intramatrical rhizoidal system; body of the spore enlarging and becoming the sporangium; spores formed within the sporangium, liberated by the bursting and dissolution of the whole distal portion of the sporangial wall; spores non-ciliate when liberated; new sporangia formed by a process of internal proliferation. Resting spores not observed.

Thraustochytrium gen. nov. Spora cellulæ matricis insidens, encystans ac systema intramatricale rhizoideum proferens; corpus sporæ se maius faciens atque sporangium exsistens; sporæ intra sporangium factæ et eruptione dissolutioneque totius partis distalis parietis sporangialis liberatæ; sporæ non ciliatæ quo tempore liberatæ; nova sporangia ratione proliferationis internæ formatæ. Sporæ perdurantes non observatæ.

T. proliferum n. sp. Sporangia smooth, piriform, very thin-walled, resting on the narrow base; 15.6–18 μ high by 10–13 μ in diameter; rhizoid branched or unbranched, of limited extent; zoöspores somewhat angular at discharge, later becoming spherical, 4 μ in diameter.

T. proliferum[3] sp. nov. Sporangia piriformia, angustæ basi insidentia, levia, pariete tenuissimo, 15.6–18 μ longa, 10–13 μ dia., rhizoidea ramosa aut non ramosa extensionis parvæ; zoösporæ angulosiores quo tempore emissæ, serius sphæroideiores exsistentes, 4 μ dia.

The developmental features of this interesting fungus (Text Figs. 22–24) (Plate I, Fig. 2) are in the main similar to those of *Chytridium* and *Rhizophidium*. However, in the *Thraustotheca*-like discharge of the zoöspores and in the proliferation of the sporangium it possesses two very distinctive characteristics. A further peculiarity is its method of internal proliferation. After the cleavage of the zoöspores there can generally be seen a larger protoplasmic unit occupying the narrow, basal portion of the sporangium (Text Fig. 25). Upon the bursting and deliquescence of the upper part of the sporangial wall by the enlarging zoöspores (Text Fig. 26), these bodies float away leaving only the basal protoplasm (Text Fig. 27). This enlarges (Text Fig. 28) and in time becomes a sporangium. Thus, in contrast to the proliferating sporangia of *Saprolegnia, Pythiomorpha,* etc., the fundament of the secondary sporangium of *Thraustochytrium* is delimited at the time of zoöspore cleavage in the primary body.

There can be no question of the non-ciliate character of the zoöspores at the time of discharge. However, in mounts containing the fungus occasional posteriorly uniciliate zoöspores have been observed which are of the same size as those discharged from the sporangia. Since no other chytridiaceous fungi were found on that particular material of *Bryopsis*, it is entirely possible that after a period of relative quiescence, the spores became ciliate.

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[3] I am indebted to Prof. R. C. Nemiah for the Latin diagnoses of new genera and species given in this paper.

Thraustochytrium differs from its closest relative *Rhizophidium*, in the internal proliferation of its sporangia, in its method of zoöspore discharge, and possibly in the possession of non-ciliate spores.

The fungus was found only on very disintegrated plants of *Bryopsis*, where it sometimes occurred with *Sirolpidium*, swarms of bacteria, and Protozoa.

Discussion

If we turn to a broader consideration of the organisms discussed in this paper, certain points of more general interest are apparent.

Perhaps the foremost of these is the fact that fungi are unquestionably demonstrated to be present in our marine waters. Although their occurrence has been recorded almost wholly from the vicinity of Woods Hole, it is highly probable that they will be found generally distributed in the littoral regions of the United States. While the number of species is as yet small, this does not necessarily imply that they are actually rare, either in kind or number. One need only to compare the bibliographies of fresh-water and marine fungi to appreciate the small amount of investigation that has thus far been accorded the latter group. Undoubtedly, more intensive work will reveal many hitherto unrecorded species.

These preliminary investigations would seem to indicate that the fungous flora in the vicinity of Woods Hole does not differ materially from that found on the other side of the Atlantic. Seven of the 13 fungi collected by the writer in the littoral of the Kattegat have been found at Woods Hole. Further indications of a probably widespread distribution of marine fungi are to be found in *Sirolpidium*, now known from the Gulf of Naples, the Kattegat (Denmark), and Woods Hole, in *Petersenia Andréei*, known from King Charles Land (Arctic), Greenland, Denmark, the Adriatic, and Woods Hole, and in *Eurychasmidium*, known from the North Sea near Edinburgh, the Baltic, and Woods Hole. It is also probable that a fungus from the Adriatic termed *Ectrogella Licmophoræ* by Scherffel (1925) is referable to *E. perforans* Pet., in which case that parasite would now be known from the Adriatic, the Baltic, and the vicinity of Woods Hole. The species of *Labyrinthula* reported by Renn (1934) as being associated with the wasting disease of *Zostera* has been observed by the present writer in diseased material of this plant from the Kattegat (1933), from Woods Hole (1934), and from Plymouth Harbor, England (1935). The organisms from these localities were morphologically indistinguishable. Future work will undoubtedly further corroborate this marked similarity of the floras which is even now so apparent.

Another point of interest is the abundance in the sea of so-called chytridiaceous fungi which possess biciliate zoöspores. While those inhabiting fresh-water algæ are, in the main, uniciliate species (true chytrids), in marine algæ, biciliate forms are more frequently found. From experience in investigating both types of habitat, the writer is inclined to believe that this is a real difference and not an apparent one resulting from the disproportionate amount of work thus far done in the two groups.

As to the question of the abundance of any one species and of the extent to which by its parasitic activities it actually limits the occurrence of a " producer," very little is now known. Of the marine fungi observed by the writer, *Ectrogella perforans* produces the most extensive infection. Indeed, a closer examination of this disease might reveal that, in some instances, it is responsible for the partial or complete disappearance of the diatom from a locality. While it is true that the two diatoms most susceptible to this fungus are littoral types (*Licmophora abbreviata, Striatella unipunctata*), it is not known whether similar epidemics may be produced by *Ectrogella* among pelagic species. In this connection, certain of the peculiar rhythms of " flowering periods " of pelagic diatoms which have been noted in the past and which cannot be attributed to changing physical factors might well be considered from the standpoint of the presence of parasites. Due to the importance of diatoms in their rôle of " producers " in the sea, such a disease as that caused by the *Ectrogella* should be examined in all its aspects, even though at the moment it appears to be confined to littoral algæ. Further, it would be well in the future to give closer attention to the examination of plankton samples for evidences of parasitic organisms, especially during the decline of a " flowering period."

In the formation of " marine humus," as Waksman (1933) has so aptly termed certain types of bottom deposits, future work may show that fungi as well as bacteria play a significant part. It will be recalled that in bottom samples taken in Great Harbor, Woods Hole, during a heavy flowering period of diatoms, two types of fungi were found in some abundance on *Rhizosolenia spp.* (*Labyrinthula* and *Rhizophidium*). Both of these " reducers " were attacking diatoms in all stages of decomposition and many of the algæ must, from their well-organized contents, have either recently fallen to the bottom or had been infected while still alive. Efforts were made to determine whether or not infected diatoms could be observed in samples from surface tows. While such indeed were found, from the nature of the waters in the vicinity of Woods Hole, subject as they are to strong tidal forces, these cannot be taken as proof of infection under pelagic conditions. It is just as possible that they were swept from the bottom as a result of tidal action. Work on the frequency of occurrence of these bottom fungi will be necessary before any estimate can be made of the extent to which they contribute to the general cycle of decomposition in the "humus."

In considering these fungi from the standpoint of their parasitism, we find a wide variation among them. Certain ones (*Ectrogella perforans, Eurychasmidium tumefaciens, Petersenia sp.* in rotifer eggs, (?)*Olpidium sphacellarum, Pleolpidium marinum, Chytridium megastomum* and *Rhizophidium* (?)*globosum* on *Bryopsis*) were true parasites and attacked, unassisted by other organisms, healthy host plants. None of these was ever observed living saprophytically. Other species (*Rhizophidium discinctum* and *Petersenia Andréei*) were only doubtfully parasitic, whereas, *Labyrinthula Chattoni*(?), *Thraustochytrium proliferum, Rhizophidium globosum*(?) on *Rhizosolenia,* and *Petersenia sp.* on *Ceramium* were saprophytes and were found only on dead algal cells. Until it can be shown experimentally that the *Labyrinthula* on decaying diatoms is the same as that parasitic on *Zostera,* it seems better to regard the two as distinct species. *Sirolpidium bryopsidis* and *Chytridium polysiphoniæ* were facultative saprophytes. It might be interpolated here that, while *Pontisma lagenidioides* was observed to be only saprophytic in the vicinity of Woods Hole, in Denmark the fungus was found to be parasitic as well, and hence it should be classed with *Sirolpidium* and *Chytridium polysiphoniæ* as a facultative saprophyte.

It is a pleasure to acknowledge at this time the continued interest of Dr. S. A. Waksman in the progress of these investigations. The writer is also indebted to members of the Botanical Staff of the Marine Biological Laboratory, particularly to Dr. H. Croasdale, for bringing in material of certain algæ.

SUMMARY

In the foregoing paper are described fifteen species of phycomycetous marine fungi, two myxomycetes, and a *Protomyxa*-like protozoan, all collected in the vicinity of Woods Hole, Mass. Included in these are two new genera and two new species. With one exception, the fungi were either parasites, facultative saprophytes, or saprophytes on various species of marine algæ. One was found parasitizing the eggs of a microscopic animal, probably a rotifer. The results of preliminary at-

tempts to culture *Sirolpidium bryopsidis,* a facultative saprophyte of the green alga, *Bryopsis plumosa,* are reported. None of these fungi, with perhaps the exception of *Rhizophidium discinctum,* has heretofore been known from the United States. The existence of true marine fungi, their work as one of the several types of "reducers" in the sea, and their possible importance in the formation of marine humus are discussed in particular.

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OCCURRENCE AND DISTRIBUTION OF THE MARINE PROTOZOAN SPECIES IN THE WOODS HOLE AREA[1]

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Investigations of the marine species of Protozoa and of their regional, seasonal, and vertical distribution and numbers are few although much descriptive attention has been given to certain groups. Such are the studies of the Radiolaria collected by the Challenger and other expeditions; the work of Brady (1884) and Rhumbler (1900) on the Foraminifera; or the papers of Schaefer (1923, 1924, 1926) on the marine amœbas. The marine Mastigophora have received scant attention as a whole although Lohmann (1902) and Schiller (1926) have published important papers. The Dinoflagellata, however, have been extensively investigated, notably by such workers as Kofoid and Swezy (1921), and Lebour (1925). There is even less general work on the marine Ciliata, although the Tintinnoidea have been extensively studied by some workers, notably Jorgensen (1927), and there are descriptive papers by Fauré-Frémiet (1924) and Kahl (1928). The " Nordische Plankton " presents a survey of the marine Protozoa, and the chapter on pelagic plant life by Gran (1912) in " The Depths of the Ocean " gives a generalization of what is known with regard to the pigment-bearing forms. How little we actually know of the occurrence and functions of the Protozoa in the seas is indicated by the fact that in the book " The Marine Plankton," (Johnstone, Scott and Chadwick, 1924) the Protozoa are not considered except for the " vegetable plankton," i.e., peridinians, whereas it has been definitely shown that a considerable part of the plankton both by numbers and volume may consist of Protozoa.

They are probably more numerous and more important than is generally realized. Gran (1912) recorded more than " 200,000 calcareous flagellates per liter . . . just outside the European coast bank " and Allen (1919) reported 14,450 per liter of sea water. He used the hand centrifuge to concentrate the organisms but was dissatisfied with the method, so by diluting his sea water into culture media and counting his cultured organisms, he estimated that the population of the sample was at least 464,000 per liter, and says " The real number must be greater than this." Only about two-fifths (178,000) of this population were Protozoa, but any such number must exert a considerable influence on the other organisms in the same water. On the other hand, as Allen pointed out, his estimated number of Protozoa may have been too small since his media were not particularly favorable for the growth of these animals.

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[1] Journal Series Paper, New Jersey Agricultural Experiment Station, Department of Water and Sewage Research.

[2] Contribution No. 65.

If the marine Protozoa are as abundant as these two instances indicate, they certainly merit more careful investigation as plankton components. Clemens' (1933) North American Pacific Coast check list includes 300 species; Smith (1904) noted 54 species from the Gulf of Mexico; Calkins (1902) studied 74 species from the Woods Hole area. Gran (1933) gave the numbers present and distribution of 4 species of flagellates, 22 of dinoflagellates, and 9 of ciliates from the Gulf of Maine; Cushman (1908) found 28 species of Foraminifera in the Woods Hole region; Martin (1929) listed 35 species of dinoflagellates from the New Jersey coast. Very little seasonal distribution has been studied in North American waters, but this phase has been investigated in the North Sea and the English Channel by Cleve (1899, 1900), Gran (1912), Herdman and Scott (1908–15), Lebour (1917) and others. From June first through early September, 1934, I studied the occurrence and distribution of the Protozoa of the Woods Hole Area.

Procedure

Studies were principally confined to those organisms occurring in water taken at the wharf of the Oceanographic Institution; the water is so turbulent there because of the strong tidal current sweeping through the Hole between Buzzards Bay and Vineyard Sound that this point samples both and there is hardly a difference in the vertical distribution of the plankton. Samples were also taken from the Bay and the Sound at various times with occasional samples from more distant points. Swimming and floating forms were secured by filtering or centrifuging samples of water varying from 120 cc. to 5 liters. A plankton net was often used to determine what larger species were present but was useless for counting purposes although relative numbers could be determined by examining the catch. Bottom-dwelling forms were secured by suspending traps from the float and by core sampling devices; later in the summer a small core bottom sampler was constructed.

TABLE I

The genera and species of marine Protozoa identified from the Woods Hole area

	Floating	Attached or in Debris	From Bottom Samples
CLASS Sarcodina			
SUBCLASS Rhizopoda			
ORDER Proteomyxa			
Labyrinthula sp.		X	
Vampyrella sp.	X	X	
Nuclearia simplex		X	
Arachnula impatiens	X	X	
Proteomyxa sp.		X	X
Biomyxa vagans		X	X
Foraminifera			
Pulvinulina menardii		X	X
Pulvinulina lateralis		X	X
Rotalia sp.		X	X
Spiroloculina sp.		X	X
Biloculina elongata		X	X
Polystomella striatopunctata		X	X
Miliolina seminulum		X	X
Miliolina circularis		X	X
Amoebaea			
Pelomyxa sp.			X
Vahlkampfia limax	X	X	X
Vahlkampfia albida	X	X	X
Amœba spp.	X	X	X
Hartmanella hyalina	X	X	X
Testacea			
Allogromia lagenoides		X	
Allogromia ovalis		X	X
Shepheardiella sp.		X	
Amphitrema sp.		X	
Pseudodifflugia gracilis		X	
Actinopoda			
Heliozoa			
Oxnerella maritima		X	
Eleorhanis sp.	X	X	
Raphidiophrys sp.		X	
Wagnerella borealis		X	
Pinnacocystis sp.	X	X	
Actinophrys sol	X	X	
Actinophrys sp.	X	X	
Clathrulina elegans	X		
CLASS Mastigophora			
SUBCLASS Phytomastigoda			
ORDER Chrysomonadida			
Chrysapsis agilis	X	X	
Chromulina minor	X		
Chromulina mikroplankton	X		
Chromulina sp.	X		
Hymenomonas roseola	X		
Erythrymonas haltericola	X		
Kephyrion ovum	X	X	X
Ochromonas variabilis	X		
Ochromonas ludibunda	X		
Pontosphaera huxleyi	X		
Syracosphæra sp.	X		
Calyptrosphæra oblonga	X		
Mesocena sp.	X		
Dictyocha fibula	X		
Dictyocha staurodon	X		
Distephanus speculum	X		
Distephanus crux	X		
Distephanus crucis	X		
Cryptomonadida			
Cryptomonas erosa	X		
Chilomonas paramecium	X	X	
Chlorachne desmorpha	X	X	
Rhodomonas baltica	X		
Rhodomonas pelagica	X		
Rhodomonas sp.	X		
Nephroselmis marina		X	
Dinoflagellida			
Exuviælla lima	X		
Exuviælla compressa	X		
Prorocentrum dentatum	X		

TABLE I (*Continued*)

	Floating	Attached or in Debris	From Bottom Samples
CLASS			
Mastigophora (cont.)			
SUBCLASS			
Phytomastigoda (cont.)			
ORDER			
Dinoflagellida (cont.)			
Prorocentrum scutellum	X		
Prorocentrum triangulatum	X		
Prorocentrum micans	X		
Peridinium depressum	X		
Peridinium roseum	X		
Peridinium achromaticum	X		
Peridinium trochoideum	X		
Peridinium mite	X		
Peridinium pallidum	X		
Peridinium inermes	X		
Peridiniopsis asymmetricum	X		
Ceratium tripos	X		
Ceratium tripos var. *atlantica*	X		
Ceratium tripos var. *atlantica* forma *baltica*	X		
Ceratium tripos var. *atlantica* forma *neglecta*	X		
Ceratium tripos var. *subsalsa*	X		
Gonyaulax triacantha	X		
Gonyaulax scrippsiæ	X		
Gonyaulax spinifera	X		
Gonyaulax digitale	X		
Dinophysis norvegica	X		
Dinophysis rotundata	X		
Dinophysis acuminata	X		
Dinophysis homunculus	X		
Dinophysis caudata	X		
CLASS			
Mastigophora (cont.)			
SUBCLASS			
Phytomastigoda (cont.)			
ORDER			
Dinoflagellida (cont.)			
Dinophysis ovata	X		
Phalacroma roseum	X		
Phalacroma rotundatum	X		
Diplopsalis lenticula	X		
Glenodinium trochoideum	X		
Oxyrrhis marina	X		
Amphidinium longum	X		
Amphidinium operculatum	X		
Amphidinium punctifera	X		
Amphidinium crassum	X		
Amphidinium fusiforme	X		
Gymnodinium punctatum	X		
Gymnodinium minor	X		
Gymnodinium achromaticum	X		
Gyrodinium fissum	X		
Gyrodinium pingue	X		
Polykrikos barnegatensis	X		
Polykrikos sp.	X		
Phytomonadida			
Pyramimonas reticulata	X		
Pyramimonas sp.	X	X	
Polytoma papillatum	X	X	
Polytomella sp.	X		
Collodictyon triciliatum	X		
Pedimonas minor	X	X	X
Chlamydomonas mikroplankton	X		
Chlamydomonas reinhardii	X		
Chlamydomonas sp.	X		

TABLE I (*Continued*)

	Floating	Attached or in Debris	From Bottom Samples
CLASS			
Mastigophora (cont.)			
SUBCLASS			
Phytomastigoda (cont.)			
ORDER			
Phytomonadida (cont.)			
Sphærella atlantica	X		
Sphærella marina	X		
Chlorogonium euchlora	X	X	
Hyalogonium acus	X	X	
Phacotus sp.	X	X	
Euglenidae			
Euglena gracilis	X		
Euglena sp.	X		
Trachelomonas euchlora	X		
Eutreptia viridis	X	X	
Euglenopsis sp.	X	X	
Astasia lagenula		X	X
Astasia sp.		X	X
Urceolus sabulosus		X	X
Peranema trichophorum		X	X
✓ *Petalomonas carinata*		X	X
Petalomonas mediocanellata		X	X
Tropidoscyphus octacostatus		X	X
Distigma urceolaris		X	X
Heteronema acutissimum	X	X	X
Heteronema sp.		X	X
Notosolenus orbicularis	X	X	X
Notosolenus apocamptus		X	X
Anisonema ovale		X	X
Anisonema vitrea		X	X
Anisonema truncatum		X	X
Dinema grisoleum		X	X
Chloromonadida			
Thaumatomastix setifera	X	X	X
Gonyostomum depressum	X	X	
CLASS			
Mastigophora (cont.)			
SUBCLASS			
Zoomastigoda			
ORDER			
Pantastomatida			
Multicilia lacustris	X	X	
Actinomonas sp.	X	X	
Cercomastix sp.		X	X
Mastigamœba longifilum		X	X
Mastigamœba socialis		X	X
Mastigella mitens		X	X
Bodopsis sp.		X	
Cercobodo crassicauda		X	X
Cercobodo longicauda		X	X
Cercobodo bodo	X	X	X
Cercobodo sp.		X	
Protomastigida			
Dallingeria drysdali	X	X	X
Monosiga ovata		X	
Salpingœca minuta		X	
Salpingœca sp.		X	
Bicocœca oculata		X	
Amphimonas globosa	X	X	X
Monas socialis	X	X	
Monas vivipara		X	X
Monas dangeardii		X	X
Bodo globosus		X	
Bodo lens		X	
Bodo ovatus		X	
Rhynchomonas sp.	X	X	X
Ancyromonas contorta	X	X	
Dinomonas vorax	X	X	
Pleuromonas jaculans		X	X
Oicomonas termo		X	X
Oicomonas socialis		X	
Polymastigida			
Tetramitus sp.	X	X	X
Hexamitus sp.	X	X	X
Trepomonas agilis	X	X	X

TABLE I (*Continued*)

	Floating	Attached or in Debris	From Bottom Samples
CLASS			
Infusoria			
SUBCLASS			
Ciliata			
ORDER			
Holotrichida			
Holophrya discolor		X	X
Trachelocerca papillosa		X	X
Trachelocerca phœnicopterus		X	X
Trachelophyllum sp.		X	X
Lacrymaria lagenula	X	X	X
Lacrymaria olor		X	X
Blepharostoma pigerrima		X	X
Chænia elongata		X	X
Chænia teres		X	X
Prorodon teres		X	
Mesodinium rubrum	X	X	
Mesodinium pulex	X	X	
Lionotus cygnus		X	
Lionotus fæciola		X	
Loxodes rostrum		X	
Chlamydodon memosyne		X	X
Chilodon sp.	X	X	
Chilodon cucullulus	X	X	X
Nassula oblonga		X	
Nassula aurea		X	
Dysteria pusilla		X	
Dysteria armata		X	
Trochilia sigmoides		X	X
Ægyria olivia		X	X
Ægyria roseus		X	
Urocentrum turbo	X	X	X
Loxocephalus granulosus	X	X	
Uronema marina	X	X	
Frontonia leucas	X	X	
Glaucoma sp.	X	X	
Colpoda sp.	X	X	
Colpidium sp.	X	X	
Cinetochilum margaritaceum	X	X	X
Paramecium sp.		X	
Pleuronema chrysalis		X	
CLASS			
Infusoria (cont.)			
SUBCLASS			
Ciliata (cont.)			
ORDER			
Holotrichida (cont.)			
Cyclidium glaucoma	X	X	X
Lembus pusillus	X	X	
Lembus infusionem	X	X	X
Lembus punctifera		X	
Plagiopyla nasuta		X	
Drepanomonas sp.		X	
Heterotrichida			
Folliculina ampulla		X	
Folliculina elegans		X	
Climacostomum sp.		X	
Condylostoma patens		X	X
Saprodinium sp.	X	X	
Oligotrichida			
Halteria grandinella	X	X	
Labœa spp.	X	X	
Strombidium sulcatum	X	X	
Strombidium grande	X	X	
Strombidium caudatum	X	X	
Tintinnopsis beroidea	X		
Tintinnopsis sp.	X		
Amphorella sp.	X	X	
Hypotrichida			
Peritromus emmæ		X	X
Oxytrichia pelionella		X	
Oxytrichia fallax		X	
Stylonichia mytilus		X	X
Epiclintes radiosa		X	
Epiclintes ambiguus		X	X
Epiclintes retracta		X	X
Amphisia kessleri		X	
Holosticha rubrum	X	X	X
Holosticha pernix		X	
Holosticha multinucleata		X	
Stichotricha secunda		X	X
Histrio pelionella		X	
Uroleptus pisces	X	X	
Uronychia setigera	X	X	
Uronychia transfuga		X	
Euplotes vannus		X	

TABLE 1 (*Continued*)

	Floating	Attached or in Debris	From Bottom Samples
CLASS			
Infusoria (cont)			
SUBCLASS			
Ciliata (cont.)			
ORDER			
Hypotrichida (cont.)			
Euplotes harpa		X	
Euplotes patella		X	
Diophrys norvegicus		X	X
Diophrys excavatum		X	
Diophrys appendiculatus		X	
Aspidisca hexeris		X	X
Aspidisca costata	X	X	X
Aspidisca turrita		X	
Onychaspis polystyla		X	
Onychaspis leptaspis		X	

	Floating	Attached or in Debris	From Bottom Samples
CLASS			
Infusoria (cont.)			
SUBCLASS			
Ciliata (cont.)			
ORDER			
Peritrichida			
Vorticella spp.		X	
Rhabdostyla spp.		X	
Cothurnia spp.		X	
Zoothamnium spp.		X	
Vaginicola sp.		X	
SUBCLASS			
Suctoria			
Acineta tuberosa		X	
Acineta sp.		X	
Ephelota coronata		X	
Ephelota sp.		X	
Podophrya fixa		X	

By centrifuging known volumes of sea water and carefully decanting, the catch was concentrated for examining living animals. A Spencer binocular microscope with apochromatic equipment and a mechanical stage were used for this. A 60X water immersion objective proved very valuable, but the metallic mounting was quickly toxic to various Protozoa. Because a Sedgwick-Rafter cell did not permit careful examination or counting of small cells, several drops of a well mixed catch delivered by a calibrated pipette were studied for each sample. The only preserved samples studied were those secured on cruises.

Many forms were found which were already familiar as fresh water species, but many others were observed which could not be traced to previously described species. When one of these unfamiliar or apparently undescribed forms appeared in numbers and on more than one occasion an effort was made to study it as a new species.

GENERA AND SPECIES PRESENT

Table 1 lists 264 genera and species of Protozoa identified from the collections made during the summer of 1934. It will be noted that some groups are very scantily treated. Thus little attempt was made to identify the several species of amœbas which were found, and the few Radiolaria which appeared in the catches about the end of the summer were likewise not identified. No parasitic forms were studied and very little attention was paid to the Suctoria.

The Sarcodina were the least important group in variety and actual numbers found. The Ciliata formed a large and varied group on the bottom and in debris but only a few occurred as free swimming or floating forms. Dinoflagellida and Silicoflagellida were numerous but curiously very few Coccolithophora appeared. Colorless, often large, Euglenida were abundant in samples containing debris and at the bottom. The Pantastomatida were very common. Samples often swarmed with extremely large numbers of small flagellated Protozoa laborious to count and identify which were usually examined to see if they could be identified as *Bodo, Dallingeria* or such small species and were then treated simply as " small flagellates." Altogether it is the Flagellata which are of most importance as swimming and suspended forms.

Of the major groups of Protozoa as listed by Calkins (1926), the Woods Hole waters contain numerous species of each in abundance. This includes the nine free living orders of Mastigophora, and all orders of Infusoria, while the only order of the Sarcodina for which representatives were not identified is the Mycetozoa.

SEASONAL SUCCESSION

No great pulses of Protozoa were found comparable to the well-known succession of diatoms in the sea. Thus no group of Protozoa ever attained the volume content of *Chætoceros* or *Rhizosolenia* at any observed time in the summer of 1934. These large diatoms more than compensated by their size for any discrepancy in numbers between themselves and the small flagellates, for example.

Practically the only Protozoa showing any succession were the dinoflagellates and silicoflagellates. Radiolaria appeared in small numbers in late August and early September but most of the Sarcodina and Mastigophora and all of the Infusoria were present to some extent at all times. *Distephanus speculum* was the sole representative of its group in early June but later its numbers decreased and other genera and species of silicoflagellates appeared. *Tintinnopsis beroidea* varied greatly in numbers throughout the summer but some were always present. Very few dinoflagellates were present early in the summer but later they became the dominant forms. Thus *Ceratium tripos* was first observed June 24, but soon thereafter it was common, and the same was true of *Peridiniopsis.* The maximum for dinoflagellate species and numbers was about mid-August. No swarming of dinoflagellates occurred either, such as that observed by Martin and Nelson (1929) in Delaware Bay. Table II gives the numbers and seasonal occurrence of various groupings of Protozoa at Woods Hole in the summer of 1934.

Table II

Estimated numbers of Protozoa per liter of sea water showing seasonal occurrence for each of four species, five related groups and totals of all Protozoa occurring at a given sampling point in the Hole at Woods Hole in the summer of 1934.

Date	*Exuviaella lima*	*Prorocentrum micans*	*Distephanus speculum*	*Tintinnopsis beroidea*	Heliozoa	Euglenoid Flagellida	Total Dinoflagellida	Total Flagellida	Total Ciliata	Total Protozoa
June 14...	1,500	375	7,875	1,500	—	—	13,875	25,500	1,875	27,375
" 16...	312	—	3,750	1,662	—	—	7,187	27,874	2,599	30,500
" 19...	1,270	—	3,810	—	—	—	5,715	115,555	300	115,855
" 21...	450	—	6,750	1,575	—	225	9,900	29,025	1,800	33,300
" 22...	1,575	—	4,725	5,250	—	—	8,400	27,825	5,250	33,075
" 23...	2,520	840	1,680	3,360	—	—	11,660	28,206	3,570	31,750
" 24...	5,940	—	3,780	1,080	—	—	13,500	58,320	1,620	59,940
" 27...	3,500	—	3,000	1,000	—	—	5,000	52,500	1,500	54,000
July 3...	5,500	4,000	8,500	8,500	—	3,000	23,000	53,750	12,500	69,250
" 10...	8,500	1,000	3,500	7,000	3,000	2,500	28,500	83,000	9,500	95,900
" 18...	6,500	2,750	2,750	3,250	3,250	3,250	15,500	90,250	4,750	100,500
" 31...	6,750	6,375	1,500	9,375	—	300	36,275	93,500	31,875	125,375
Aug. 8...	1,500	—	500	2,625	—	750	16,875	68,250	8,250	76,490
" 15...	5,250	5,625	—	9,750	1,500	4,175	46,875	178,125	15,750	135,375
" 22...	7,875	3,375	—	6,375	1,875	3,750	31,875	111,720	9,375	122,970
" 29...	6,750	5,250	—	7,500	2,250	1,500	40,875	102,395	13,875	119,270

Nature and Accumulation of Sea Floor Population

The rate at which the protistan flora and fauna become established on the bottom of the sea in summer, at least in shallow waters, is amazing. Syracuse dishes were suspended from the float enclosed in wire cages so that only very small organisms could have access to them. At stated periods a dish was removed, wiped clean underneath, and its contents examined with a water immersion objective. It was soon found that direct examination was no longer possible after 24 hours; sediment and organisms were too thick to see through after that interval. Table III gives the numbers of identified organisms which accumulated in a representative dish in 24 hours between July 9 and 10.

It will be seen from Table III that the population consisted of about one-third each of Protozoa, diatoms, algæ and fungi. In this average sample the number of organisms listed is smaller than the number actually accumulating in 24 hours. Some are out of the plane of observation, some are obscured by debris, some encyst in the allotted period, and some occur which cannot be identified. Small samples of film from such a Syracuse dish were examined and the only organisms which could be found were diatoms, amœbæ, the ciliates *Cyclidium* and

Lembus and the flagellates *Bodo* and *Rhynchomonas.* These samples were then added to filtered sea water enriched with nutrient materials and gave thriving cultures of several genera of diatoms; four species of amœbas; the ciliates *Uronychia, Cyclidium, Lembus, Holosticha, Euplotes charon* and *Euplotes patella* as well as some unidentified ciliates; *Oxyrrhis marina, Amphidinium operculatum* and another unidentified dinoflagellate, *Rhynchomonas, Bodo* and *Notosolenus;* and green, brown, red and blue-green algæ, these last being especially rich in number of species and abundance. Evidently the sea bottom shelters many organisms other than those in the trophic state, and to arrive at the true population one should augment visual examination of fresh samples with a variety of cultures designed to produce excystment and growth; the cultures above were produced on only one type of medium. Instead of accumulating a population of 144,000 Protozoa per square meter in 24 hours, the sea bottom at this shallow point might acquire many times that many.

TABLE III

Numbers of protistan organisms accumulating in 24 hours per square centimeter of exposed Syracuse dish. A typical sample.

Diatoms	16,120
Small algal cells	17,980
Fungi	2,480
Unidentified amœbeæ	2,400
Unidentified Heliozoa	620
Unidentified flagellates	5,580
Unidentified ciliates	310
Exuviœlla lima	930
Gymnodinium sp.	620
Ceratium lineatus	240
Pyramimonas inconstans	1,240
Calycomonas sp.	2,480
Trachelomonas euchlora	240
Anisonema ovale	620
Notosolenus orbicularis	240
Chlamydodon memosyne	240
Cyclidium glaucoma	240
Trachelocerca phœnicopterus	240
Euplotes patella	240
Aspidisca costata	240
Podophrya fixa	240
Total Protozoa per sq. cm.	17,060

The nature of the sea bottom largely determines the nature and rate of accumulation of its population. Sandy or current-swept bottoms might have a small population, except in crevices, depressions or other sheltered situations. In the Syracuse dishes representing a sheltered bottom a film was gradually built up during four weeks greatly reminiscent of the film accumulating on the stones of the trickling filter in a sewage disposal plant. It consisted of much debris but nematodes, hydroids, and other Metazoa were abundant in it. Its chief population, however, consisted of Protozoa and diatoms with a few filamentous algæ. The deeper parts of the film contained few Protozoa, the maximum population being in the porous upper part. Wherever such a thin blanket of film as this exists on the sea floor it must be of great importance in the mineralization of organic debris, which obviously occurs to the greatest extent only where such a film can be built up. The few samples obtained in 1934 from the sandy bottom of Vineyard Sound yielded few Protozoa, but samples from the mud banks in Great Harbor showed an abundance of flagellates and ciliates. Table IV shows the Protozoa identified from two such bottom samples, together with those obtained

TABLE IV

Protozoa in the surface film of two samples from muddy bottoms, and those obtained by towing five feet off bottom where one of the mud samples was obtained.

Buzzards Bay	Great Harbor
Forty-five foot depth	Nine-foot depth
Amœbeæ, three species	Amœbeæ, two species
Foraminifera, four species	Foraminifera, two species
Gromia lagenoides	*Shepheardiella tæniformis*
Anisonema vitrea	*Anisonema vitrea*
Dinema griseolum	*Anisonema* sp.
Distigma proteus	*Heteronema* sp.
Urceolus sabulosus	*Peranema trichophorum*
Monas amœbina	*Urceolus sabulosus*
Monas obliqua	*Mastigamœba longifilum*
Bodo globosus	*Monas vivipara*
Lionotus wrzesniowski	*Bodo angustus*
Loxodes rostrum	*Polytoma uvella*
Peritromus emmæ	*Prorocentrum triangulatum*
Trachelocerca phœnicopterus	*Rhodomonas baltica*
Oxytrichia fallax	*Cyclidium glaucoma*
Uroleptus pisces	*Prorodon* sp.
Diophrys excavatus	*Chlamydodon memosyne*
Tintinnopsis beroidea	*Trachelocerca phœnicopterus*
Unidentified ciliates, three species	Unidentified ciliate, one species

Towing. Buzzards Bay

Distephanus crux
Exuvaiella lima
Ceratium fusus
Gonyaulax triacantha
Peridiniopsis asymmetrica
Monas vivipara
Strombidium sulcatum
Ochromonas marina
Exuviælla marina
Ceratium lineatus
Peridinium divergens
Phalacroma roseum
Holophrya griseum
Tintinnopsis beroidea

from a towing just off the bottom in the same locality for comparison. Bottom-dwellers are largely holozoic or saprozoic, whereas those from towings in shallow water are largely holophytic or saprozoic. The small number of species from the bottom samples is accounted for by the fact that the core of mud in each case was about 18 mm. in diameter. No fresh cores from deep samples were examined in 1934, although a core four days old from a depth of 200 meters contained numerous *Bodo* and *Anisonema* and a new species of *Prorodon.*

The Role of the Protozoa

It seems generally agreed that the Protozoa are important as a food source for other organisms in the sea, especially such animals as copepods. Examination of the gut contents of salps and copepods generally shows an abundance of diatom and dinoflagellate shells as well as those of Tintinnoidea. But such examinations reveal nothing about the use of naked dinoflagellates, and the smaller ciliates and flagellates; they disintegrate and leave no recognizable remains in the gut. I am of the opinion that these two latter types are more important in shallow waters than the shelled forms because of their abundance, and also because the large diatoms like *Chætoceros* and *Rhizosolenia,* which were exceptionally numerous at Woods Hole in 1934, cannot be ingested by the smaller copepods. Direct observation is not very practical, but small copepods were seen feeding on the bacteria and Protozoa in laboratory cultures in July and six months later were abundant in those cultures containing few diatoms but abundant bacteria and Protozoa. Clarke (1935) has proved that copepods use these minute cells as food, and from Table II it may be inferred that they are sufficiently numerous at all times in the summer to serve as food. If these very small protozoan cells are considered to be roughly spherical, an individual 5 microns in diameter would have a volume of 65.45 cubic microns, and a liter could contain roughly 15^{12} such individuals, so that a population of 300,000 per liter which has been noted at Woods Hole would still be a relatively sparse one. Nevertheless, the feeding mechanism of such animals as salps and copepods is perhaps capable of straining out these food organisms and the rapidity of cell division of these small forms permits a speedy recovery.

Nelson (1935) cites the importance of the dinoflagellates as food for oysters and has successfully experimented with the feeding of them to oysters. In areas such as the New Jersey coastal waters where the dinoflagellate population is great they may have a decided importance for this reason. Undoubtedly more careful investigation of the food of many marine animals will reveal a larger rôle of the Protozoa as food organisms.

The Protozoa also have an important function in the turnover of organic matter; this is not separable from their function as food-forming organisms. Dr. C. J. Fish remarked to me, " There must be numerous Protozoa in the surface waters; if a dead copepod is taken while over Georges Bank and allowed to stand in sea water from the same source, it is quickly attacked by a swarm of Protozoa." This is an easily made observation. Bacteria swarm about the dead animal also and they and the Protozoa penetrate inside the exoskeleton where they are not swept away by currents; there they work rapidly and soon only the chitinous shell remains. Often the visible Protozoa are so thick inside that their volume must be many times that of the decay bacteria. Direct observation is inadequate as proof that they actually ingest the flesh of the dead animal, but a *Loxophyllum* quickly becomes filled with round bodies, presumably stored food, while a *Lembus infusionem* can be seen forming food vacuoles containing ingested bacteria. The larger ciliates may feed on bacteria or on diatoms or other Protozoa and are themselves sizeable food morsels, as a species of *Chænia* and one of *Trachelocerca* each over 1,500 microns long. The holozoic and saprozoic forms attack organic debris in suspension or on the ocean floor and constantly convert it to protoplasm in part and accomplish its mineralization in part. If they do not compare with the bacteria in numbers, their size is a compensation for this.

Some Protozoa, especially the smaller ones, may be a part of the food chain by partly acting as " food condensers." Butterfield (1933) has shown that some bacteria thrive in a food medium too dilute for the ciliate *Colpidium*, which alone requires 500 mg. per liter of certain substances, but which will grow on 5 mg. per liter of these substances if *Bacillus ærogenes* is present, the bacterium apparently " condensing " the dilute food substances and being eaten in turn by the ciliate. It seems probable that the numerous smaller and presumably saprozoic flagellates may condense food in the sea in a similar manner, and in turn become food for larger forms.

The photosynthetic activities of the Protozoa are also important. The pigmented forms are numerous, and have a separate value in the food chain because of their utilization of inorganic matter. This work of those containing chlorophyll is self evident, but many brown, red and other coloring matters are found in the various groups, and the functions of these coloring matters is unknown. In the Woods Hole area pigmented Rhizopoda are principally Heliozoa and few in number, but pelagic Radiolaria containing the yellow symbiotic flagellate, *Chrysi-*

della, are abundant in the open sea. Among the flagellates four of the orders contain many pigmented forms, the most important being the Dinoflagellida. Chlorophyll is practically lacking in the marine Infusoria but other colors are not.

The elaboration of glucose and subsequent products by these pigmented forms must be evaluated in any consideration of the carbon content of the sea. Furthermore, the depths at which the pigment-bearers are found must be investigated, especially since they are active and rapid swimmers and are more independent of flotation mechanisms than the diatoms.

CONCLUSIONS

Systematic examination of the waters in the Woods Hole area has shown the existence of a large protozoan population from early June to mid-September. The population of this shallow tide-swept area is probably greater than hitherto suspected and must have a considerable ecologic importance because of its numbers, ability to reproduce rapidly, place in the food cycle, and furtherance of mineralization of organic debris. Scant examination of the off-shore waters indicates that the large inshore numbers are approximated in these off-shore waters also. Chlorophyll and other pigment-bearing forms predominate near the surface while at the bottom bacterial feeders and saprozoic forms are most abundant. Changes in the dinoflagellate population suggest seasonal succession but no such changes were observed among other types. The fauna at the bottom accumulates rapidly when protected and its members are active in the constant mineralization of the deposited debris. The maximum numbers in this film are determined by its thickness and rate of deposition. A weekly census of organisms present was kept, and a check list of the genera and species observed in the area has been made.

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EFFECT OF ULTRAVIOLET RADIATION ON THE HEART OF LIMULUS POLYPHEMUS

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The heart of *Limulus polyphemus* was excised along with the dorsal ganglion. The rhythmically beating heart was then placed in a finger bowl containing 200 cc. of sea water. Temperature changes were observed by means of a thermojunction. One blackened junction was placed on the ganglion at the level of the sixth segment. As the heartbeat of the *Limulus* is neurogenic (Carlson, 1904 and 1905, and Garrey, 1932), its frequency and amplitude depend on the action of the ganglion. Thus, if the temperature of the ganglion is recorded it is easy to determine whether frequency changes are attributable to temperature variations of the pace-maker. The source of ultraviolet employed during this investigation was a Cooper-Hewitt Uviarc, with a 6-inch tube and reflector, placed at 35 cm. from the preparation.

Figure 1 indicates the effects of repeated short intense ultraviolet radiations on the *Limulus* heart with the dorsal or ganglion surface receiving the radiation. The temperature of the ganglion is also graphed (broken line curve). It is quite apparent that the temperature increases caused by the heat of the mercury arc were not responsible for the observed changes as the increases in temprature never exceeded 0.3° C. during a 3-minute period of radiation. Furthermore, while there was a progressive rise in temperature, it was slight and was not accompanied by a corresponding progressive and sustained increase in cardiac rate; on the contrary, each brief exposure of the heart to the action of ultraviolet light was accompanied by a burst of acceleration which was followed in each instance by a recession of rhythm to the initial rate or even to a slower rhythm when the exposure ceased. The general tendency of the temperature curve is toward a higher level. This is attributable in the main to the fact that the room temperature was higher than the initial temperature of the bath although the radiation may have added, in a minor degree, to the rise of temperature of the bath.

It was observed that the amplitude of the heart's contractions also increased when these short doses were applied. No correlation between increase in frequency and in amplitude was found.

Shielding the heart with a glass plate and radiating the heart had no effect. Thus, it is concluded that rays longer than 3,500 Angstrom units are not responsible for the results reported and that it is the ultraviolet region which is the factor to be considered.

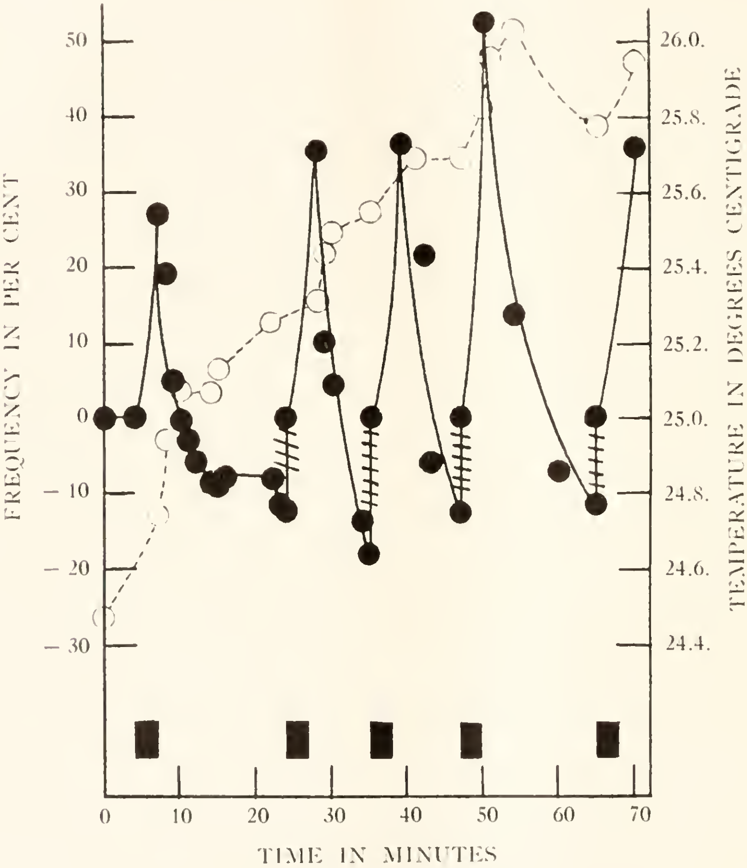

Fig. 1. Graph indicating the effect of ultraviolet light on frequency of *Limulus* heart-beat. ● frequency; ○ temperature; ■ period of radiation.

Discussion

The *Limulus* heart responds to ultraviolet radiation in a manner similar to that of the frog and alligator (Guttman, 1935). Since the *Limulus* heart-beat is neurogenic, the above experiments demonstrate the definite stimulating effect of ultraviolet light of rhythmogenic processes in nerve cells of the cardiac ganglion.

It was observed that by the end of 3 minutes of radiation the maximum increase appeared and then a rapid fall of the indices ensued. Radiation for longer periods caused a greater fall in the frequency and amplitude values after the initial increase. Hinrichs (1926) states that "the amount of radiation in a given region of the spectrum, which is absorbed by the protoplasm in a given period of time, determines whether there is to be an increase or decrease in the speed of the physiological process." Packard (1931) working with X-ray, reports "an acceleration which is followed by a retardation and often by a very obvious injury."

It may be supposed, in the case of the *Limulus* heart, that the primary action of the radiation is to increase the frequency of the nerve cell discharges and also to increase the number of active nerve cells, hence the number of nerve fibers transmitting impulses to the myocardium is increased. The former supposition would account for the increased frequency, the latter for the increased amplitude. The secondary effects (the decrease in the indices) may be attributed to a decrease in the number of fibers transmitting impulses and a decrease in their frequency.

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OXYGEN PRODUCTION BY ZOÖXANTHELLÆ IN A BERMUDAN TURBELLARIAN

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An intimate association of plants and animals such as the occurrence of Zoöchlorellæ and Zoöxanthellæ in certain protozoans, cœlenterates, and flatworms presents some interesting problems which have long attracted biologists. The most extensive studies have been made by Keeble and Gamble on the two species of *Convoluta* found at Roscoff. Many interesting observations were made by these investigators but one of the most important, the production of oxygen by the algæ as a result of photosynthesis and the subsequent use of this oxygen by the animal, was not extensively studied. It has been easier in the case of the green planarian, *Convoluta roscoffensis,* than in the case of the yellow form, *C. paradoxa,* to demonstrate that photosynthesis occurs and that the animal benefits from the presence of the algæ in its body. Geddes (1879) and Haberlandt (1891) found the green planarian when placed in darkness dies in the course of two or three days, the implication being that when photosynthesis is arrested death from starvation ensues. Geddes with a simple experiment, testing by a glowing match tip, was the first to show that the gas given off in the sunshine by this same form was oxygen. Keeble (1910), in summing up the work carried on jointly with Gamble, says that the green cells in *C. roscoffensis* are capable of photosynthesis and, when exposed to light, can utilize carbon dioxide, give off oxygen, and manufacture carbohydrates the excess of which is stored as starch. This starch in turn is used by the animal, as is shown by its disappearance in animals kept in the dark. Keeble (1908, 1910) said it was not easy to prove that the yellow-brown cells of *C. paradoxa* were capable of photosynthesis but finally concluded that the reserve food contained in these algal cells was probably a product of photosynthesis, and served the animal for nutritive purposes. He claims the reserve food is a fatty substance, in the form of droplets and is not starch, thus differing from the stored food of the green form. Since *C. roscoffensis* and *C. paradoxa* have no excretory system, Keeble states the algal cells in both forms act as excretory organs and use the waste nitrogen products of the animal.

The object of the present investigation was to determine whether a sufficient amount of oxygen might be produced by Zoöxanthellæ to be of value to flatworms harboring them and if so, to determine the quantitative relations between oxygen production and consumption in the light and in the dark. A form suitable for this work was found in abundance during the summer months, in the aquaria at the Bermuda Biological Station, where this investigation was carried on during June and July, 1933 and 1935. This turbellarian is probably *Amphiscolops langerhansii,* described by von Graff (1905), but the description by Haswell (1905) of an Australian acœlous turbellarian *Heterochœrus* is in some respects a better description of the Bermuda form, particularly of the reproductive apparatus. However, it is believed that the two forms are identical and the genus *Amphiscolops* is already well established. *Amphiscolops langerhansii* according to von Graff occurs in nature in shallow rock-pools between tidal limits on the brown seaweed and sand, but several attempts failed to locate such a source and the laboratory aquaria supplied the several hundred specimens used in the course of the work.

These turbellarians are positively phototropic and gather on the sides of the aquaria toward the light. They are bilobed at the posterior end and when not stretched out in moving, measure 2 to 3 mm. long and 1 to 1.3 mm. wide. There are two red eye spots and a statocyst easily seen in animals which have been kept in the dark and whose color is lighter due to fading and shrinking of the Zoöxanthellæ. Besides the brown color due to the many Zoöxanthellæ, there is a reflecting pigment scattered over the dorsal surface in a more or less median line.

The Zoöxanthellæ are not seen in a newly hatched larva, but appear after a few days and in the adult measure 0.020 to 0.022 mm. in diameter. These algal cells in a healthy adult worm, when stained with iodine, are seen to contain small bluish bodies which are evidently starch. When treated with 95 per cent alcohol the Zoöxanthellæ lose the yellow-brown color first, then the chlorophyll collects as light green droplets and begins to exude from the algæ as their cell walls break down and they disintegrate.

The worms were collected from the sides of the aquaria by means of a pipette and placed in 70 cc. bottles filled with sea water which had been allowed to stand for at least an hour in order to permit the escape of excess oxygen. The number of animals per bottle varied from one to thirty. The ground-glass stoppers were water-sealed in order to prevent an exchange of gases with the air, and the bottles were placed either in a dark-room or in a situation where they received bright diffuse light during daylight hours. The object was to determine whether *Amphiscolops* kept in the light would survive longer than when kept in the dark and also whether a few animals would survive longer than many animals in a limited amount of oxygen. The bottles were examined at least once each day and the number of days the worms survived was noted. All of the individuals of a given bottle died within a few hours of one another, thus making it easy to determine the number of days of survival of the group. It is possible that the first dying worms had a toxic effect on the remainder and this might account for the individuals of a given group dying so nearly simultaneously. However, the uniformly consistent results obtained in all experiments indicated that the chance death of an animal, and its possible toxic effect on the remainder could be of little significance. The results are shown in Table I. In the tests carried on in the dark it may be seen that in all cases single individuals were surviving when the experiment had to be discontinued, giving an average survival time of more than fifteen days. With five animals per bottle the time of survival was reduced to six days on the average, and a gradual reduction may be noted until for thirty animals the time was *2.2* days. Only one series of controls in the light is shown, that done in 1935. They were not all started on the same date but all were surviving at the end of sixteen or nineteen days, which was as long as it was possible to make observations. It is evident from these tests that some product of the photosynthesis carried

TABLE I

Showing time of survival of worms in sealed bottles kept in constant darkness, except during periods of examination.

No. of worms	1	5	10	20	30
No. of days survival . . .	20+ 13+ 13+ 13+ 16+	6 8 5 7 4 6	4 6 3 3 5 3	3 3 3	2 2 2 3
Averages	15+	6	4	3	2.2

Showing time of survival of controls in diffuse light, for experiments carried on during June and July, 1935.

No. of worms	1	5	10	20	30
No. of days	19+	19+	19+	19+	16+

on by the symbiotic algæ benefits the animals which harbor the algæ. To what extent it may be the oxygen produced will be seen in the experiments which are now to be discussed.

Sealed bottles containing one to thirty worms in sea water were prepared as in the survival tests and one of a pair placed in the dark, the other in bright diffuse daylight. While no attempt was made to control the temperature, it fluctuated only slightly about an average of 26° C. After the bottles had remained in the light or in the dark for two to five days and before any of the worms had begun to die, oxygen tests were made. The Winkler method according to Kemmerer, Bovard, and Boorman (1923) was used. The results may be seen in Table II. The amount of dissolved oxygen in the sea water at the beginning of

Table II

Showing the amounts of oxygen in bottles containing one to thirty worms kept for a period of days in the light and in the dark.

No. of worms	Day tested	Dark	Light
		cc./liter	*cc./liter*
1	4	3.82	4.72
1	4	4.22	3.37
1	5	3.13	4.03
1	5	3.13	
Averages	4.5	3.58	4.04
5	4	3.26	4.38
5	4	3.37	4.61
Averages	4	3.31	4.50
10	4	2.52	4.57
10	4	1.49	4.57
10	4	2.06	4.80
10	4	1.79	4.14
10	4	2.24	
10	4	2.01	4.47
Averages	4	2.02	4.51
20	2.5	1.37	4.57
Averages	2.5	1.37	4.57
30	2	.23	4.05
30	2	.67	
Averages	2	.45	4.05

the experiments ranged from 4.0 to 4.5 cc. per liter. The oxygen content of the sea water was not determined for each set of experiments. The sea water used in each of the bottles of a given pair came from the same sample, however, and the light series serves as a control, as the object was to determine the oxygen consumption in the dark. Table II gives the amounts at the end of two to five days for varying numbers of worms kept in the light and in the dark. In the light the average amounts of oxygen may be seen to vary from 4.04 to 4.57 cc. per liter, showing that under normal light conditions enough oxygen is produced by photosynthesis to supply the needs of the worms and to maintain a comparatively constant amount of dissolved oxygen.

If we now examine the results of the tests made on animals kept in the dark it may be seen that in the case of one animal the average volume of oxygen remaining at the end of four and one-half days was 3.58 cc. per liter and a gradual reduction in oxygen occurred with an increase in the number of animals per bottle, until for thirty animals only 0.45 cc. per liter remained at the end of two days. These results indicate that in the dark a group of *Amphiscolops* soon uses the limited supply of oxygen and the Zoöxanthellæ are naturally unable to replace it. In fact, in the absence of light the symbiotic algæ must be a distinct burden to the worms for they likewise utilize some oxygen and also fail to remove the carbon dioxide and other waste products of the worms.

TABLE III

Showing the difference in oxygen content of the light and dark series of Table II, which gives a relative measure of oxygen consumption in the dark. From this it is possible to calculate the oxygen consumed by one worm in a day. It is necessary to assume that in the light the oxygen content is maintained at approximately its original level by the photosynthetic activity of the algæ.

No. worms	Day tested	Total O_2 consumed	O_2/day	O_2/worm/day
		cc./liter	*cc.*	*cc.*
1	4.5	0.46	0.10	0.10
5	4	1.19	0.30	0.059
10	4	2.49	0.62	0.062
20	2.5	3.20	1.28	0.064
30	2	3.60	1.80	0.060
				Average 0.069 cc./liter

From the data of Table II it is possible to calculate the rate of oxygen consumption in the dark and this is shown in Table III. Except in the case of single worms the amount of oxygen consumed per worm per day does not vary much from the average of 0.069 cc. per liter per day. This indicates that the method employed and the results obtained are quite reliable.

In view of the fact that in daylight the dissolved oxygen is maintained approximately constant, this average of 0.069 cc. per liter also represents roughly the amount of oxygen produced per day by the Zooxanthellæ of a single worm. The symbiotic algæ of *Amphiscolops,* although yellow-brown algæ, are therefore seen to produce enough oxygen by photosynthesis to supply the needs of the worms which harbor them; thus they are just as valuable as are the Zoöchlorellæ of *Convoluta.*

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A CHROMOSOME STUDY OF THE APHID MACROSIPHUM SOLANIFOLII

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Introduction

The extensive genetic work being done in this laboratory on the cycle of *Macrosiphum solanifolii* with respect to wing production, the gamic forms, and intermediates should eventually necessitate a study of the possible chromosome relations with the cyclical phenomena. The present work, dealing primarily with the normal chromosome behavior, will serve as a basis for such an investigation. After the problem had been under investigation for some time cytological results on other species of aphids (Jeffrey, 1933, and Suomalainen, 1933) were seen to be not in agreement with previous work done mainly by Morgan (1908, 1909, 1910, 1912, 1915) and von Baehr (1908, 1909). There was thus a second object in the present study—that of throwing light on the disagreement between the new work and the old.

The problem was suggested by Professor A. Franklin Shull and throughout its progress his generous helpfulness and pertinent suggestions have been of inestimable value.

Life Cycle

Parthenogenetic wingless females of *Macrosiphum solanifolii* appear on the rose plant in the spring, presumably developing from fertilized eggs laid the preceding fall. These so-called stem mothers give birth parthenogenetically to winged or wingless parthenogenetic females. Succeeding generations are likewise both winged and wingless in variable proportions. The potato serves as the summer host. Migration to it occurs in several generations or possibly in all generations as long as any aphids are left on the rose, and some individuals may remain on the rose throughout the summer.

During the summer these two types, the winged and wingless parthenogenetic females, continue to bring forth successive generations of parthenogenetic young, the winged forms producing more wingless offspring than the wingless ones produce.

In the fall the winged forms give birth to gamic females. This may occur on the rose if the winged mother of the gamic female has migrated thither. The males, which are winged and are born mostly of wingless mothers, develop on the potato plant and are believed to seek out the females on the rose. After fertilization the eggs are laid. These eggs overwinter presumably on the rose, and from them stem mothers develop in the spring.

Material and Technique

Unlimited numbers of parthenogenetic females of the potato aphid were easily maintained throughout the entire year on potato plants grown in flower pots in the laboratory. The males occurred in smaller numbers and at long intervals so that whenever they did appear it was necessary to preserve for future study as many as could be handled.

For a study of the maturation stages of the parthenogenetically developing egg, the adult parthenogenetic wingless females were fixed and sectioned, and studies were made of the ovarioles within the enclosed embryos. The maturation stages in the males were most numerous in the second and third instars.

Navashin's fixative (4 to 12 hours) gave the best results on both chromatin and cytoplasm and was used almost exclusively. The N-butyl alcohol technique for animal tissues described by Stiles (1934) was used for dehydration and infiltration. The insects were embedded in paraffin; sections were cut at 7 micra and stained in Heidenhain's iron hematoxylin.

The drawings of cell outlines were made with the aid of a camera lucida and a Zeiss 2 mm. (1.3) apochromatic objective and a 15 × ocular. These outlines were later enlarged with an opaque projector and details of cells drawn free-hand.

Spermatogenesis

Spermatogonial Mitosis

The testes are bilobed structures composed of a number of cysts containing germ cells in various stages of development. The germ cells in any particular cyst are in the same stage of development and in the embryos these cells are all spermatogonia, many of them undergoing mitotic division.

The resting spermatogonia (Fig. 1) have a characteristic large, rounded nucleus with a heavily staining nucleolus, which in the majority of cases lies near the nuclear membrane. The cytoplasm surrounding the nucleus is a comparatively thin layer and contributes little to the size of the cell.

The chromatin first appears in the early prophase as a network of fine, lightly staining threads connecting heavily staining granules scattered throughout the nucleus. The threads appear single, though it is impossible to determine this with certainty. As the chromatin threads condense they become grouped about the nucleolus, radiating in long loops from this point. The completion of the condensation in late prophase reveals nine chromosomes (Fig. 2), all of which are rod-shaped and of the same thickness, but of five different lengths. The longest chromosome is unpaired. The rest are paired; the longest of the four is slightly shorter than the single chromosome, the smallest is nearly spherical, and the others are of intermediate lengths. The nucleolus is still present at this time but it becomes smaller as prophase proceeds and eventually disappears just before the appearance of the spindle.

At metaphase the chromosomes arrange themselves normally on the equatorial plate, but become so closely crowded together that they are individually indistinguishable. The chromosomes divide longitudinally and, retaining their compact, crowded form, pass as two groups of daughter chromosomes to the poles. Interzonal fibers are evident at this time extending from one group of chromosomes to the other. After completing a short resting stage the cells divide again.

Meiosis

After completion of growth as normally occurs in meiosis the pri-

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EXPLANATION OF PLATE I

Fig. 1. Spermatogonium in resting stage.

Fig. 2. Prophase of spermatogonium showing nine chromosomes. The longest is the unpaired chromosome.

Fig. 3. Primary spermatocyte in resting stage.

Fig. 4. Prophase of primary spermatocyte showing paired chromatin threads. Nucleolus in center.

Fig. 5. Prophase of primary spermatocyte with chiasma-like pairing of chromatin threads.

Fig. 6. Later prophase. One of the paired chromosomes in the center of the nucleus is broken and the broken ends attached to the ends of one of another pair of chromosomes.

Fig. 7. Diakinesis showing nine chromosomes. Eight of the chromosomes are paired and one is unpaired.

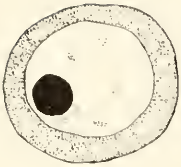
Fig. 8. Polar view of equatorial plate of first meiotic division.

Fig. 9. Lateral view of equatorial plate. Unpaired chromosome in center of spindle with ends directed toward poles.

Fig. 10. Anaphase. Unpaired chromosome lengthened and extending from one group of separating chromosomes to the other.

Fig. 11. Later anaphase showing constriction of unpaired chromosome at same level as cytoplasmic constriction.

Fig. 12. Telophase. Cytoplasm divided unequally by constriction. The unpaired chromosome extends from one nucleus to the other.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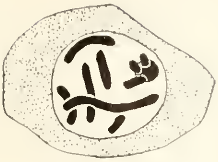
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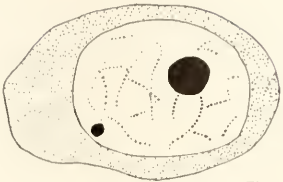
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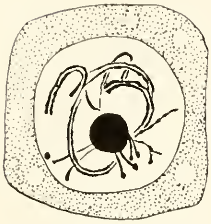
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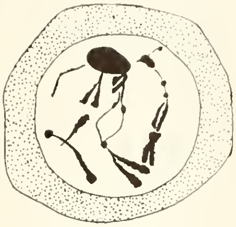
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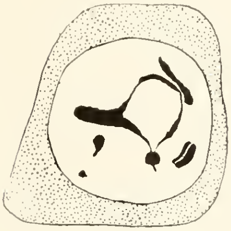
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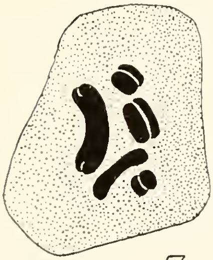
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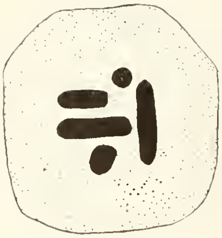
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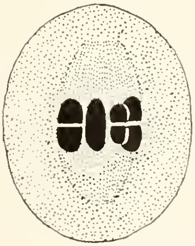
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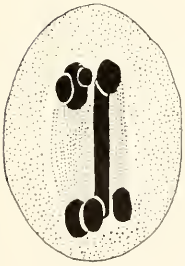
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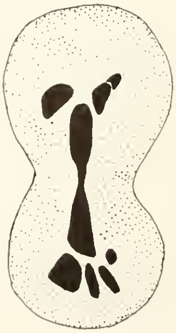
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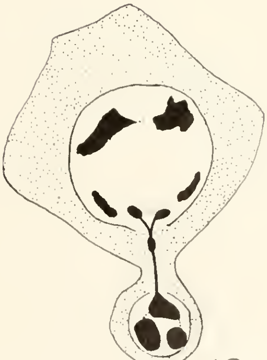
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PLATE I

mary spermatocytes (Fig. 3) are about twice the size of the spermatogonia. One other difference between the two types of cells is the presence of a small nucleolus lying near the nuclear membrane of the primary spermatocyte in addition to the large nucleolus, which is like that found in the resting stage of the spermatogonia.

The prophase development is normal. The chromatin threads first appear as faint, beaded threads connecting rather large chromatin bodies (about half the size of the small nucleolus) that are scattered throughout the nucleolus. These granules disappear as condensation continues, leaving distinctly double, roughly beaded chromatin strands, which arrange themselves in loops extending outward from the nucleolus (Fig. 4).

The prophase stages showing the paired chromatin threads were studied with particular care in the hope of finding any unusual rearrangement of the chromatids. The segregation of genetic characters has never been determined definitely in any specific pattern in aphids due to the difficulty of breeding the gamic forms, though Shull (1925) found indication of a multiple factor inheritance of color. Therefore any prophase configurations that might indicate crossing over or other rearrangements of the chromatids, and thus genetic characters, would be of particular interest in this respect.

The intimate union of the chromatids at certain points, while keeping distinctly separate between these points, is particularly characteristic of chiasma formation. Recent cytological studies indicate that the formation of chiasmata is definitely correlated with crossing over so that the presence of one is evidence of the other. In Fig. 5 is shown a cell in which the paired chromatids have the appearance of characteristic chiasmata. A second example of chromatid rearrangement is shown in Fig. 6. One chromatid of a pair has become broken in the middle, the broken ends becoming attached to the ends of one chromatid of another pair. This might very easily result in a type of translocation. It is thus evident that there is in the prophase of the male maturation division the mechanism whereby portions of chromosomes can and do become rearranged, and which presumably results in a rearrangement of genetic characters.

The further contraction of these prophase strands ultimately forms smooth, rod-shaped chromosomes of unequal lengths (Fig. 7). They are thicker and shorter at this stage than in the corresponding stage of spermatogonial mitosis, and the size relationship between the individual chromosomes varies in one respect from those of the preceding division, namely, that in the spermatogonial mitosis the unpaired chromosome is the longest, while in the diakinesis of the first maturation division the unpaired chromosome is exceeded slightly in length by one of the other pairs.

A difference of size relationship of the unpaired chromosome to the other chromosomes in the cell between the prophase of the spermatogonial mitosis and the first maturation division was described for one other aphid (*Euceraphis betulæ*) by Shinji (1931). He states: "Here [prophase of the first maturation division] the X-elements, four in number, are individually and collectively smaller than any one of the bivalents in the spermatocytes. When, however, we examine the equatorial plate of the spermatogonia . . . each X-element, except one, is nearly as large as any of the autosomes."

The arrangement of the chromosomes on the equatorial plate is normal in every respect. The unpaired chromosome is attracted to the plate to the same extent as the bivalents, and differs in this respect from the usual behavior of unpaired chromosomes in other forms, and in particular those of hybrids, in which the unpaired chromosomes are scattered over the spindle during metaphase and are not attracted to the plate until after the separation of the bivalents. A polar view shows only 5 chromosomes on the equatorial plate at this time, inasmuch as only 4 of the paired chromosomes and the unpaired chromosome are visible (Fig. 8). The unpaired chromosome is the second largest on the plate, though its identity can only be inferred from a knowledge of its size relation to the other chromosomes. This equatorial plate is a stage more often found than any other in the entire maturation and while the arrangement of the chromosomes differs occasionally, the unpaired X-chromosome is always on the plate. I believe, therefore, that it can be said with a high degree of assurance that the unpaired chromosome is subject to the same forces and to the same degree of regulation as the bivalents, and does not behave in an irregular manner. In a side view of the same stage there is no difficulty in determining the unpaired chromosome (Fig. 9). In the particular cell here drawn, the unpaired chromosome has further contracted and turned so that it lies perpendicular to the other chromosomes on the plate, and each end is directed toward one of the poles, while the paired chromosomes are just on the point of separating. The spindle fibers are visible but there are no centrosomes, which seems to be characteristic of all the cell divisions in this particular species.

The force exerted upon the paired chromosomes during anaphase, to draw them to the poles, must attract the two ends of the unpaired chromosome to the same degree as the bivalents, for each end of this chromosome is drawn toward one of the poles along with the segregating bivalents. This necessitates a stretching and lengthening of the unpaired chromosome (Fig. 10), which seems as though it were destined to divide transversely in half along with the equal division of the cell, for a constriction appears in the middle of the lengthened chromosome and at the same level a constriction appears in the cytoplasm (Fig. 11).

The cytoplasm does not divide into two equal parts, for immediately after the appearance of the constriction it begins to slip toward one end of the elongated cell, and at the final division only a small amount of the cytoplasm is pinched off. This unequal division produces secondary spermatocytes of two sizes of which one is a large cell containing most of the cytoplasm and the other is a small cell containing very little cytoplasm (Figs. 12, 13, 14, 15).

The constriction of the lagging chromosome appears first halfway between the two ends, but gradually, as the cytoplasmic constriction slips to one end of the cell, the entire middle region of the chromosome becomes compressed while the ends get slightly larger as though part of the chromatin material were being forced into the ends by the constriction. At this time the lagging chromosome is much like a dumb-bell in appearance. Each end of the unpaired chromosome lies within the newly formed nuclear membrane of the daughter cells while the thin constricted middle portion extends through the cytoplasmic bridge that connects the two cells before division is complete (Figs. 12 and 13). The formation of the nuclear membrane is not complete in either cell at this time, for a gap is left open in both nuclei through which the lagging, unpaired chromosome extends (Fig. 12).

The appearance of the lagging chromosome at this time might easily lead one to conclude that it divides transversely, half going into each daughter cell. This does not occur, for just before the small cell is pinched off by the cytoplasmic constriction, the lagging chromosome is withdrawn from the small cell and goes in its entirety into the large cell (Fig. 14). Examples of this lagging chromosome going into the large

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EXPLANATION OF PLATE II

Fig. 13. Telophase.

Fig. 14. Later telophase. Greater part of unpaired chromosome in larger cell.

Fig. 15. Large secondary spermatocytes having five chromosomes and small secondary spermatocytes having four chromosomes. Chromosomes split longitudinally in preparation for second meiotic division.

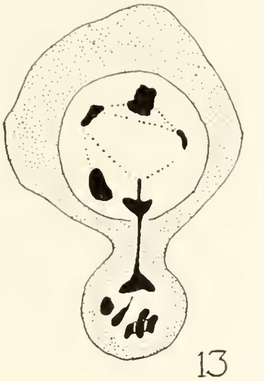
Fig. 16. Polar view of equatorial plate of second meiotic division.

Fig. 17. Anaphase of second meiotic division.

Fig. 18. Spermatids. Three degenerating, small secondary spermatocytes lying among spermatids.

Fig. 19. Later spermatid.

Fig. 20. Mature spermatozoa. Only a part of the tail has been drawn extending to the left.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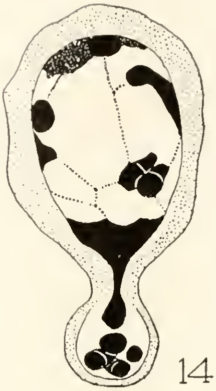
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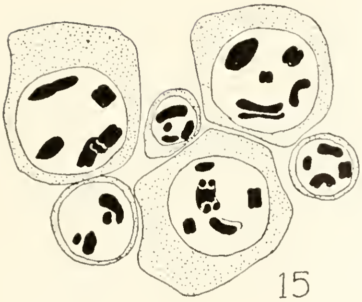
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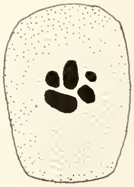
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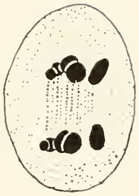
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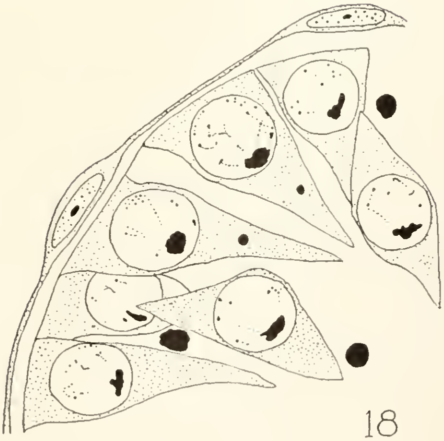
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Plate II

cells are very scarce, due no doubt to the rapidity with which the event takes place, though it is not difficult to find stages immediately before and after the final movement of this chromosome.

In the cell drawn in Fig. 14, showing the lagging chromosome going into the larger cell, the chromosomes are larger than those in the drawings of the preceding stage (Figs. 12 and 13). This might cause some question as to the accuracy of interpreting Fig. 14 as the stage following Figs. 12 and 13. The difference in size is probably not due to any fundamental difference between the two stages nor to a growth of the chromatin in the later stage, as might be supposed from these drawings, but rather to the swelling effect of the fixative used on the aphid from which Fig. 14 was taken. Gilson's fluid was used on this aphid and in general seemed to cause greater swelling than Navashin's fixative which was used on the aphids from which Figs. 12 and 13 were taken.

Immediately after the lagging chromosome has been drawn into the large cell, the small cell is severed completely from it by the cytoplasmic constriction. The two types of cells remain side by side throughout the cyst for some time. The large cells contain most of the cytoplasm and 5 chromosomes, while the small cells contain very little cytoplasm and only 4 chromosomes (Fig. 15).

After completion of the first division the large cells go through a short resting stage or interphase in which the chromatin becomes indistinct. Even in early telophase before the first division is complete the chromosomes begin to lose their well rounded, smooth appearance (Figs. 12, 13, and 14). The chromosomes spread out, seem to dissolve, and finally disappear altogether. The chromosomes in the large cells in Figs. 12 and 13 show this to some degree, but the best example is the chromosome lying along the periphery of the nucleus opposite the lagging chromosome in Fig. 14. The beaded or granular chromatin threads connecting the chromosomes, which were particularly evident during prophase of spermatogonial mitosis, are present again at this time. These connecting threads are characteristic of this aphid, for in some cases I have even observed them extending from one chromosome to the other on the equatorial plate of the first meiotic division, and they are particularly evident whenever the chromosomes are not condensed to their fullest extent, whether this be during prophase or during telophase. Stevens (1905) described similar connecting threads in *Aphis œnotheræ*.

The chromosomes in the large cells split longitudinally before the interphase, this being the first indication of the second maturation division which occurs immediately after the interphase. It seems likely that this split condition is retained through this resting stage, though during it the individual chromosomes become lost to view, for at the first appearance of the chromatin threads in their recondensation their double nature is apparent. This return of the chromatin to a condensed condition follows normal prophase behavior, the chromatin becoming evident first as faint, thin threads which gradually thicken and shorten until they become typical chromosomes. There is a greater contraction at this time than during the prophase of the first meiotic division for the chromosomes that assemble on the equatorial plate of the second meiotic division are about one-half their former size (Fig. 16). However, all the chromosomes contract to the same degree so that each chromosome in any particular cell has the same size relative to the other chromosomes in that cell as in the preceding division. A direct comparison in one microscopic field of cysts containing equatorial plates of the second meiotic division with cysts containing the same stage in the first division shows clearly that the entire cell at the metaphase of the second division is smaller than the same stage in the first division.

During the anaphase of the second meiotic division (Fig. 17) the chromosomes divide along the longitudinal splits that were first visible in telophase of the preceding division before the interphase. This division is equational, none of the chromosomes lag, and each of the two equal cells that are formed contains 5 chromosomes, of which 4 are autosomes and one the unpaired or, as it has been termed by others, the X-chromosome. Interzonal fibers that stained no more densely than the spindle fibers are evident during the anaphase of this division. They extend from one group of separating chromosomes to the other.

After completion of the second maturation division the large cells develop into spermatids (Figs. 18 and 19) and eventually into mature spermatozoa (Fig. 20).

The small secondary spermatocytes remain scattered throughout the cysts among the large cells for some time after the first maturation division. The chromosomes split longitudinally and remain in the centers of the cell as if about to arrange themselves on the equatorial plate, but no spindle is formed and cell division never goes beyond this point. Instead the chromosomes become clumped together, while the cytoplasm begins to degenerate and gradually disappears, leaving a round mass of chromatin that stains very heavily. After the second maturation division the majority of the degenerating cells are found near the periphery of the cyst where they presumably have been forced by the movement of the large secondary spermatocytes during the division. The number of degenerating cells gradually decreases as development proceeds. A few are still evident lying among the developing spermatids (Fig. 18), but before the development of the mature spermatozoa the last trace of the small secondary spermatocytes is gone.

The presence of these two types of cells makes it possible to determine whether every division of a primary spermatocyte results in two unequal cells or whether two equal secondary spermatocytes are occasionally produced from one division. If in any one cyst after the first maturation division the number of large cells equals the number of small cells, the division of every primary spermatocyte must have been unequal. And since only the large cells divide in the second division we should expect to find after that division that the spermatids were twice as numerous as the degenerating cells. If the division of the primary spermatocytes were not always unequal but occasionally resulted in two equal daughter cells, the above ratios would not be expected.

TABLE I

Counts of male germ cells after the first and second maturation divisions showing the relative frequency of the two types of cells.

AFTER FIRST MATURATION DIVISION

Cyst	Large secondary spermatocytes	Small secondary spermatocytes
1	132	130
2	20	19
3	64	61
4	190	189
5	72	72
6	108	105
Total	586	576

AFTER SECOND MATURATION DIVISION

Cyst	Spermatids	Degenerating cells
1	372	212
2	235	94
3	263	108
4	302	166
5	408	201
6	467	236
Total	2047	1017

Counts were made of the secondary spermatocytes immediately after the division of the primary spermatocytes while each small cell was still lying close to its large partner. The results are shown in Table I. In the upper half of this table are listed counts of the large and small cells of 6 cysts directly after the first maturation division. In each cyst, as in the total of the entire 6 cysts, the large cells and the small cells are about equally numerous. The proportion of the large cells in the total is 0.5043 ± 0.0146 (standard error, not probable), which is an insignificant deviation from 50 per cent. The lower half of the same table shows the counts made after the second maturation division. There is a greater variation in the individual cysts from the theoretical 2:1 ratio, but of the total number in the 6 cysts the proportion of large cells is 0.6680 ± 0.0085, which is only insignificantly different from two-thirds.

These proportions are so close to the expected ratios that there can be little doubt that the primary spermatocyte always divides unequally, resulting in large and small secondary spermatocytes in equal numbers, and that the large cells divide again, eventually producing viable spermatozoa, while the small cells degenerate. Schwartz (1932) reported similar results from a study of *Tetraneura ulmi* de Geer.

Maturation of the Parthenogenetic Female Egg

The reproductive system of the parthenogenetic female consists of a vagina, two short oviducts branching from the vagina, and a variable number of ovarioles (up to ten) branching from the oviducts. At the end of each ovariole is a germarium in which the germ cells are found.

Maturation begins in the germarium and is completed in the upper end of the ovariole to which the germarium is attached. The reproductive system of the most fully developed embryos enclosed within the adult parthenogenetic females proved to be the best place in which to study the maturation stages. The germaria within the embryos are made up of yolk-producing cells and germ cells, while each ovariole contains a maturing oöcyte and an embryo in the blastula stage of development. The oöcyte lies in the anterior end of the ovariole close to its attachment to the germarium and the embryo is found just posterior to the oöcyte. In Fig. 21 is shown a longitudinal section of a germarium and the end of the ovariole containing an oöcyte undergoing maturation. The embryo which usually lies posterior to this oöcyte is not shown. The germarium represented in the upper half of the drawing is separated from the oöcyte by a constriction of epithelial cells which form a covering for the germarium and make up the wall of the ovariole. These epithelial cells form a structure much like the finger of a glove of which the germarium occupies the extreme tip.

The germarium is an ellipsoidal (sometimes nearly spherical) structure in which are yolk-producing or so called " nurse " cells, and germ cells. The nurse cells are at the extreme tip of the ovariole and form a single-layered ball of cells surrounding a cavity filled with yolk, much like a typical bastula (Fig. 21).

Directly beneath the nurse cells in the drawing are shown 3 oögonia, two of which are in a prophase stage of oögonial mitosis, while the third, the center one, is beginning to increase in size and develop into an oöcyte. The oögonial divisions occur in this region of the germarium, but after each oögonium has completed its last mitotic division its descendant cells begin to grow and pass through the neck of epithelial cells into the ovariole. This epithelial membrane is elastic so that the opening is widened, allowing the enlarged germ cell to descend into the ovariole. The growth of the oöcyte is presumably due to the direct flow of yolk into the cell from the source of supply within the ball of nurse cells. A stream of yolk passes from this yolk supply to the oöcyte, and follows it down through the neck of epithelial cells into the ovariole, remaining connected until after the completion of maturation. In Fig. 21 this yolk stream can be seen extending from the yolk supply through the neck of epithelial cells to the tip of the ovariole. The drawing does not show a direct connection between the yolk stream and the oöcyte, but this can be plainly seen in Fig. 24. At the top of the latter drawing are represented a few of the epithelial cells which are a continuation of the ovariole wall and form a connection between the ovariole and the germarium. Between these two layers of cells is the yolk stream, flowing directly into the oöcyte by way of an opening in the cell membrane.

After entering the ovariole, the oöcyte continues to increase in size, as does the nucleus, and eventually becomes ellipsoidal in shape, due perhaps to the pressure exerted by the walls of the ovariole. It is impossible to count the individual chromosomes at this stage as they are wound about one another in a compact mass in the center of the nucleus (Fig. 21). After the oöcyte attains a size slightly larger than the germarium the nucleus migrates to the periphery, usually midway between the two ends of the ellipsoidal oöcyte. After reaching this point the nuclear membrane disappears and the individual chromosomes arrange

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EXPLANATION OF PLATE III

Fig. 21. Upper half—germarium of parthenogenetic female showing cross-section of ball of nurse cells with yolk in center. Yolk stream extending from yolk supply to tip of ovariole. Three oögonia directly beneath nurse cells. Lower half—tip of ovariole with primary oöcyte.

Fig. 22. Primary oöcyte. Polar view of equatorial plate.

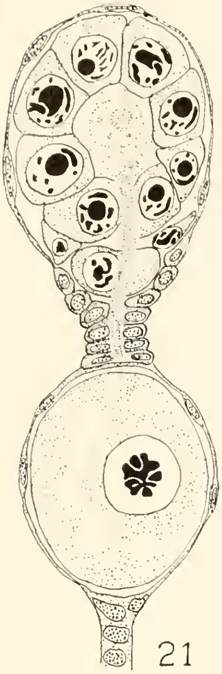
Fig. 23. Primary oöcyte. Lateral view of equatorial plate.

Fig. 24. Early telophase of maturation division and formation of polar body. At top of figure yolk stream entering oöcyte.

Fig. 25. Mature parthenogenetic egg with cleavage nucleus in resting stage. Polar body at right of figure between embryo and ovariole.

Fig. 26. Polar view and lateral view of cleavage mitosis in developing embryo.

Fig. 27. Polar view of somatic mitosis in male.



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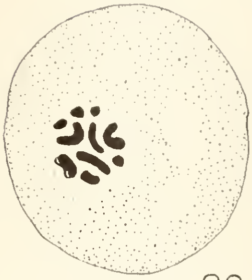

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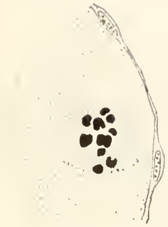

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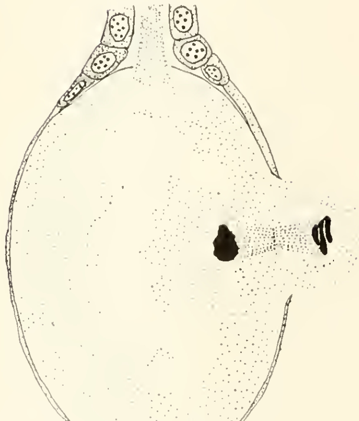

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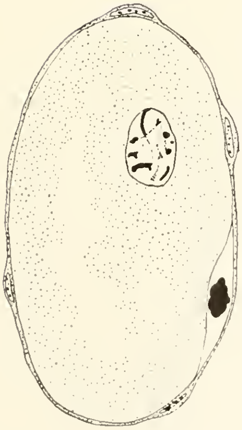

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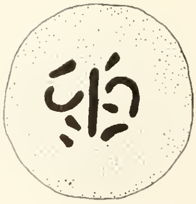

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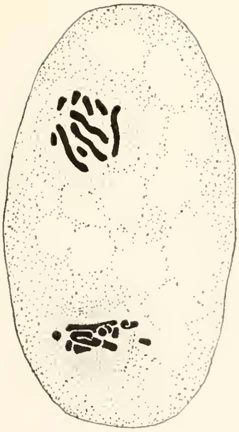

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PLATE III

themselves on the equatorial plate of the spindle (Figs. 22–23). In Fig. 22 ten chromosomes, each slightly furrowed at the ends indicating a longitudinal split, are visible from a polar view of the equatorial plate. A side view of the spindle shows one pole lying close to the periphery of the oöcyte and the other pole directed toward the center of the egg. Ten chromosomes are also visible here, and in fact in all my observations of complete equatorial plates of this division, particularly the polar views, I never observed any variation of this number. The poles of the spindles lack centrosomes, as is true also of the cell divisions in the males. The chromosomes divide along the longitudinal splits previously noted, 10 chromosomes being extruded in the form of a polar body while 10 remain in the egg. A late anaphase of this division with characteristic interzonal fibers extending from one group of chromosomes to the other is shown in Fig. 24. It is impossible to count the individual chromosomes at this time because of their crowded condition. The polar body is pinched off and comes to lie between the ovariole wall and the egg membrane, as is shown in Fig. 25. The small amount of cytoplasm that surrounds the chromosomes of the polar body at the time of its separation from the egg has by this time degenerated and a small, darkly staining mass of clumped chromatin is all that is left. The polar body later sinks into the cytoplasm of the egg and is evident in the cytoplasm of the developing embryo until about the fourth or fifth cleavage division, after which time it is no longer visible, possibly having been absorbed.

As soon as the maturation division is complete and the polar body is extruded a nuclear membrane is formed around the chromosomes that remain in the egg. The chromosomes lose their identity as the resting stage of the first cleavage nucleus is formed, which remains near the periphery of the egg and soon undergoes the first cleavage mitosis. These first cleavage divisions are nuclear only, each of the daughter nuclei remaining close to the periphery of the egg so that a syncytium is formed of a peripheral row of nuclei around a central mass of clear cytoplasm. The cytoplasm eventually divides between the nuclei, forming a single layer of peripheral cells which usually surrounds two or three nuclei that are lying free in the central area of cytoplasm.

The individual chromosomes are very distinct in polar views of cleavage mitoses, which show ten chromosomes of five different sizes (Fig. 26). The five different sizes of chromosomes have the same size relationships as those described for the male germ cells, and also for the somatic cells of the male, as in Fig. 27 in which is shown an equatorial plate of a somatic mitosis taken from the wing bud of a male. The chromosomes of the female cleavage divisions differ from the male germ and somatic chromosomes in that one member of the largest pair of chromosomes in the female is absent in the male. The absence of this chromosome in the male is the cause of the unpaired condition of one of the chromosomes of the male, which lags in maturation. Thus the condition in this species of aphid, *Macrosiphum solanifolii,* is similar to that found in phylloxerans by Morgan and for other aphid species by von Baehr and others in that the female has one more chromosome than the male. This chromosome and its partner, which form the longest pair in the female, are the so-called X-chromosomes. Two of them are found in the female while only one is found in the male.

Gamic females were not available for a study of their chromosomes, but it is safe to assume from analogy with other species of aphids that the chromosome number in the gamic female is 10 or the same as in the parthenogenetic female and that eggs are produced having the haploid number of 5 chromosomes. This assumption can be made with a high degree of certainty because in all the species previously studied the results have been the same. The disagreement has always been concerned with the fate of the lagging chromosome in the male. Because of the degeneration in the male of the secondary spermatocytes containing 4 chromosomes, all the viable spermatozoa contain 5 chromosomes and the individual that develops from the fertilized egg of the gamic female must necessarily contain 10 chromosomes, which is the female number.

Discussion

The controversy in aphid cytology has invariably been concerned with the fate of the unpaired chromosome or chromosomes, and the relative sizes and fate of the secondary spermatocytes. Morgan (1908), von Baehr (1908), Schwartz (1932), and others claimed that the unpaired chromosome acted as a sex-determining chromosome and went in its entirety into one secondary spermatocyte, while the other secondary spermatocyte receiving very little cytoplasm, was much smaller and degenerated. Jeffrey (1933) believed the previous work to be in error, stating that in *Aphis salicicola* and *Aphis populifoliæ* the division of the primary spermatocyte resulted in two cells of equal size, neither of which degenerated. The chromosomes were assorted equally between the two secondary spermatocytes and while he mentioned the presence of lagging chromosomes, which were similar to lagging chromosomes described previously by other workers, he did not refer directly to their behavior. He held that the lagging chromosome indicated the existence of hybridism in the aphid. Suomalainen (1933) likewise disagreed with the work previously done for he thought that the lagging chromosome divided transversely, a small portion of one end being pinched off by the cytoplasmic constriction during the division of the primary spermatocyte. However, he agreed with Morgan and others that small secondary spermatocytes were formed which degenerated, and that the lagging chromosome acted as a sex-determiner. Suomalainen made no mention of Jeffrey's work.

My own work on *Macrosiphum solanifolii* is in direct opposition to that of Jeffrey. In this species there is an unequal assortment of chromosomes as the extra, unpaired, lagging chromosome goes wholly into one of the secondary spermatocytes, resulting in this secondary spermatocyte having one more chromosome than the other. The cytoplasmic division of the primary spermatocytes is also unequal so that secondary spermatocytes of two sizes are produced. The larger cells have the greater number of chromosomes and develop into mature spermatozoa, while the small secondary spermatocytes degenerate.

The only point in which Suomalainen's results differ from mine is that of the fate of the lagging chromosome. He states that it divides transversely, a small piece of one end being left in the small secondary spermatocyte. Suomalainen observed a small piece of darkly staining material in the small secondary spermatocyte which he assumed to be the pinched off end of the lagging chromosome. However, he was uncertain of this for he states: ". . . allerdings ist es schwer mit absoluter Sicherheit festzustellen, ob es wirklich ein Chromosom ist, da das Plasma auch andere mit Hämatoxylin sich dunkel färbende Körper enthält." In *Macrosiphum solanifolii* the small secondary spermatocytes contain no structure than can be interpreted as the pinched off end of the lagging chromosome.

Suomalainen states as further proof of the division of the lagging chromosomes that the chromosomes of the second maturation division are smaller than those of the first maturation division. The reduction in size is presumably not a result of division, however, for I find a similar shrinkage in *Macrosiphum solanifolii* and here the lagging chromosome goes entirely into the large cell and does not divide.

In order to overcome the genetical consequences of the loss of one end of a chromosome, Suomalainen assumes that the end of the lagging chromosome is an "Eliminationchromosom," by which he may mean that it is inert, while the part that goes into the large cell is the active part, that is, contains the genes. No active portion of the lagging chromosome can be regularly pinched off to degenerate in the small secondary spermatocyte, for if the lagging chromosome lost one end during meiosis there would be a deletion in the next generation. If this shortened chromosome again found its way into a male in the next gamic phase of the cycle, as it normally would in 50 per cent of the males produced, it would suffer a further deletion during the maturation of the germ cells. With a continuation of this process, which would be inevitable in the normal chromosome cycle, the lagging X-chromosome would eventually disappear.

There are two means by which such a loss could be avoided, by fusion during fertilization of the large part of the X-chromosome furnished by the spermatozoön with a small part of the same type of chromosome which would be furnished by the egg, or by the regeneration of the lost end during the parthogenetic phase. The fusion with the large piece of the X-chromosome of a small piece of the same type of chromosome during fertilization is improbable, for a study of the entire chromosome cycle reveals no mechanism whereby such a small piece of chromatin could be produced. Regeneration of inert chromatin may be possible though that this could occur with chromatin containing active genes seems unlikely, and it is unknown whether the end of the lagging chromosome is active or inert. If regeneration of a part of a chromosome can occur, Suomalainen's interpretation of the dividing X-chromosome could be correct, otherwise it is questionable.

The conclusions resulting from this work are in complete accord with the early work of Morgan (1908–1915), von Baehr (1908–1909) and the more recent work of Schwartz (1932).

Summary

In *Macrosiphum solanifolii* the male has nine chromosomes, while the parthenogenetic female has ten chromosomes, the male lacking one chromosome of the longest pair present in the female.

During the spermatogonial mitosis the nine chromosomes divide equationally and none of the chromosomes lag.

Certain prophase configurations during spermatogenesis indicate a strong possibility of crossing over.

Four pairs of chromosomes and one unpaired chromosome are visible during diakinesis of the first maturation division in the male.

The unpaired chromosome is attracted to the equatorial plate along with the bivalents at metaphase, differing in this respect from univalents in hybrids.

The unpaired chromosome lags and becomes lengthened at anaphase while the bivalents are being drawn to the poles, and remains extended across the plane of division as much in one daughter cell as in the other until just before the cytoplasmic constriction separates the two cells completely, at which time the unpaired chromosome goes in its entirety

into one of the cells. Thus of the two cells produced by the first division, one contains five chromosomes and the other contains four.

The cytoplasmic division of the primary spermatocyte is also unequal, as the greater proportion of the cytoplasm goes into the secondary spermatocyte containing the five chromosomes. The secondary spermatocyte with only four chromosomes receives very little cytoplasm and is about half the size of the other.

The large secondary spermatocytes with five chromosomes divide again after a short interphase. The division is equational and none of the chromosomes lag, so that each of the two cells resulting from this division contains five chromosomes. These cells develop into spermatids and eventually into mature, viable spermatozoa.

The small secondary spermatocytes with four chromosomes do not divide again and eventually degenerate.

The large and small secondary spermatocytes are present in equal numbers after the first maturation division, which indicates that the division of every primary spermatocyte is unequal. After the second maturation division the large cells, which at this time are developing into spermatids, are twice as numerous as the small, degenerating cells.

The parthenogenetic female has ten chromosomes. Only one polar body is given off during maturation, and as the division is equational, ten chromosomes go out with the polar body and ten remain in the egg.

A comparison of the somatic chromosomes of the male with those in the parthenogenetic female shows that the male lacks one chromosome of the longest pair present in the parthenogenetic female.

Gamic females were not available for this study, but on analogy with other aphids they should contain ten chromosomes.

The conclusions resulting from this investigation support Morgan's work on phylloxerans in opposition to that of Jeffrey and Suomalainen.

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FURTHER EXPERIMENTS ON THE FEEDING OF CALANUS FINMARCHICUS[1]

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INTRODUCTION

The prominent position of copepods in the food cycle of the sea has caused various investigators to study conditions influencing their production—in particular their food supply. Since previous observations on the food relationships of copepods were meager and conflicting, a series of experiments was initiated at the laboratory of the Woods Hole Oceanographic Institution in an effort to throw light upon this problem (Clarke and Gellis, 1935).

In their previous work these investigators obtained most satisfactory results with *Calanus finmarchicus.* It was found that individuals provided with sea water which had been passed through filter paper lived and moulted in large numbers, whereas other individuals, provided with sea water from which all particulate matter had been removed by means of a membrane filter, failed to moult and eventually died. Since the copepods throve in water from which the larger constituents of the phytoplankton—chiefly diatoms—had been removed, it was evident that this species at least could live on the small pelagic organisms, or nannoplankton, and perhaps actually did so in nature.

The further experiments on *Calanus* about to be described were undertaken in order to test the whole question more thoroughly, and, it was hoped, to ascertain at the same time which of the many components of the nannoplankton are chiefly concerned. There exist in the sea many bacteria, unicellular algæ, and protozoans which are small enough to pass through the pores of the rather coarse filter paper used (Whatman No. 12). Since bacteria were expected to be numerically most abundant, we began our scrutiny of the nannoplankton by testing this group as a possible source of food for *Calanus.*

EXTENSION OF EXPERIMENT USING CONTINUOUSLY FLOWING MEDIA

In the first of the present experiments, it was proposed to control more carefully than previously the bacterial population present in the flasks by using measured amounts of known strains of bacteria and by keeping careful track of subsequent changes in the population. Using the same method as Clarke and Gellis, continuously flowing media were supplied to flasks in which copepods were confined. One flask received water filtered through a Berkefeld cylinder of porosity W (which was found to remove all bacteria and to be more convenient than the membrane filter used previously). A second flask received Berkefeld-filtered water which had been inoculated with a small amount of a mixed suspension of three types of common marine bacteria. A third flask was supplied with a large amount of the same bacteria in Berkefeld-filtered water. At the end of four days bacterial counts showed that the first flask did not remain sterile and the desired quantitative difference between the second and third flasks was not maintained. Since the bacterial population in the flasks was not satisfactorily controlled, a different procedure was obviously necessary, but the experiment was useful in demonstrating the extreme rapidity with which the bacterial situation can alter in culture flasks.

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[1] Contribution No. 92, Woods Hole Oceanographic Institution.

ATTEMPTS TO FREE COPEPODS OF BACTERIA

The difficulties cited above would have been avoided, had it been possible to remove all bacteria from the copepods before commencing the experiment. Attempts to wash the animals free of bacteria by passing through the flask in which they were confined about sixty liters of water in eight hours resulted in a reduction of the bacterial count from over 10,000 to under 10 per cc., provided the animals were transferred to a fresh sterile flask twice during the washing period. Encouraged by this great reduction, we constructed a special washing tube consisting of a test tube (12.5 × 2.8 cm.) to the bottom of which was sealed an elbow of small bore glass tubing (Fig. 1). A glass bead allowed water to enter from below, but prevented the escape of any copepods when the flow of water ceased. Sea water collected at about three hundred yards from shore was passed through filter paper and then through the Berkefeld filter. The continuous flow, which was maintained from the filter, was divided into two parts each of which ran through a cooling coil and then was introduced at the bottom of one of the special washing tubes suspended in a water bath at 12° C. The upper end of the washing tube was closed with a two-hole rubber stopper through which passed (1) an outflow tube, the mouth of which was covered with bolting cloth, and (2) a second tube containing a cotton plug, which was kept closed until the end of a run, when it was used to equalize the pressure in the washing tube with that of the atmosphere. The outflows of the two washing tubes joined and were connected by way of two carboys to an aspirator which drew water through the whole system. A negative pressure of from 440 to 340 mm. of mercury as measured by a manometer in the system was maintained during the washing. Tests showed that this reduced pressure had no harmful effect upon the copepods.

Filter, cooling coils, washing tubes, and outflow tubes were sterilized in an autoclave. Twenty *Calanus* were introduced into each tube and washed for two or three hours with twenty liters of sea water. At the end of the washing periods the animals were poured into flasks of sterile water cooled to 12° C. This water had been passed through a Berkefeld filter, and then heated in the flask for thirty minutes at 90° C. Bacterial counts made on these flasks shortly after adding the copepods amounted to from 0 to 12 bacteria per cc. After standing overnight

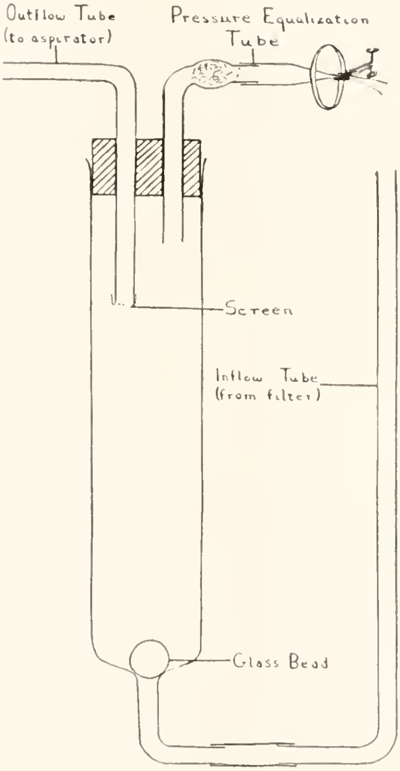

FIG. 1. Washing tube for removing bacteria from *Calanus*.

the counts rose to from 60 to 400 per cc. This concentration of bacteria we believe to be negligible in so far as the nutritional needs of copepods are concerned (see below).

The Availability of Bacteria in High Concentrations as Food for Calanus

Four lots of twenty animals each were washed daily by the method described above. Two of these groups, No. 1*a* and 1*b*, were each poured after washing into a 250-cc. flask containing 150 cc. of sterile water prepared as described above. Lot No. 2 was placed in paper-filtered sea water. Lot No. 3 was placed in water treated as for groups 1*a* and 1*b*, but subsequently inoculated with a loopful of bacteria[2] scraped from an agar slant. A bacterial count was made on each flask after the copepods had occupied it for approximately twenty hours. The animals were then transferred to the washing tube by means of a sterile wide-mouthed pipette. The copepods in all four groups were subjected to equal manipulation which did not appear *per se* to cause injury. A considerable number of animals were lost, however, by being caught on the sides of the tube during pouring. In reporting the results of the experiment these animals are not considered (Table I).

The numbers of bacteria in these flasks varied considerably, but were consistently very much smaller in flasks No. 1*a* and No. 1*b* than in flasks No. 2 and No. 3. During the first nine days both flasks No. 1*a* and No. 1*b* gave counts close to 500 per cc. Subsequently the counts rose markedly to average between 2,000 and 4,000 bacteria per cc., and on one day (forty hours after washing) were about 10,000 per cc. in each flask. In flask No. 2 the counts ranged from 50,000 to 800,000 per cc. and averaged 250,000. Bacterial counts in flask No. 3 fluctuated from 300,000 to 1,500,000 per cc. with an average of 600,000. From the nutritional point of view, flasks No. 1*a* and 1*b* may be considered sterile because, as will be shown below (Table III), the relatively small number of bacteria present would provide a negligible amount of nutriment. The concentration of bacteria in flask No. 3 was many times greater than has ever been found in the sea. In flask No. 2 small flagellates were present also, but time did not permit a quantitative estimation to be made.

The apparent paradox of the highest death rate occurring in the flasks best supplied with food may possibly be explained by the fact that in flask No. 2 six of the deaths (50 per cent) and in flask No. 3 two of the deaths (20 per cent) occurred during moulting, whereas in flasks No. 1*a* and 1*b* there were no indications of moulting. The copepods in flasks No. 2 and 3 were apparently receiving enough food to carry them to the moulting stage, but not enough to enable them to pass this critical period. It is possible that in flask No. 3 *Calanus* did not utilize the bacteria in suspension, but rather fed on the small amount of solid matter unavoidably added with the inoculum. The starved animals lived longer on the average than those with a little food, because they did not grow, and did not attain any critical stage. Since in the experiments of Clarke and Gellis *Calanus* moulted successfully when supplied with paper-filtered water, the attendant conditions must have been more favorable in some respect. Possibly more food was made available by the use of continuously flowing water.

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[2] These bacteria were contributed by Dr. Selman A. Waksman. They belonged to a common marine species listed as No. 18 in his collection.

TABLE I

Bacteria in high concentrations as food for Calanus. Percentage of animals living after number of days indicated at left. Twenty animals at start.

	Flask No.	1*a*	1*b*	2	3
	Treatment of sea water	Sterilized		Paper filtered	Bacteria added
	Accidental deaths	7	6	7	8
days					
0		100	100	100	100
3		80	100	100	88
6		64	100	87	75
9		57	93	69	67
12		54	93	38	50
15		54	64	8	33
18		31	43	8	17

Two conclusions may be drawn from this experiment:

(1) *Calanus* will live without growing for over two weeks under starvation conditions.

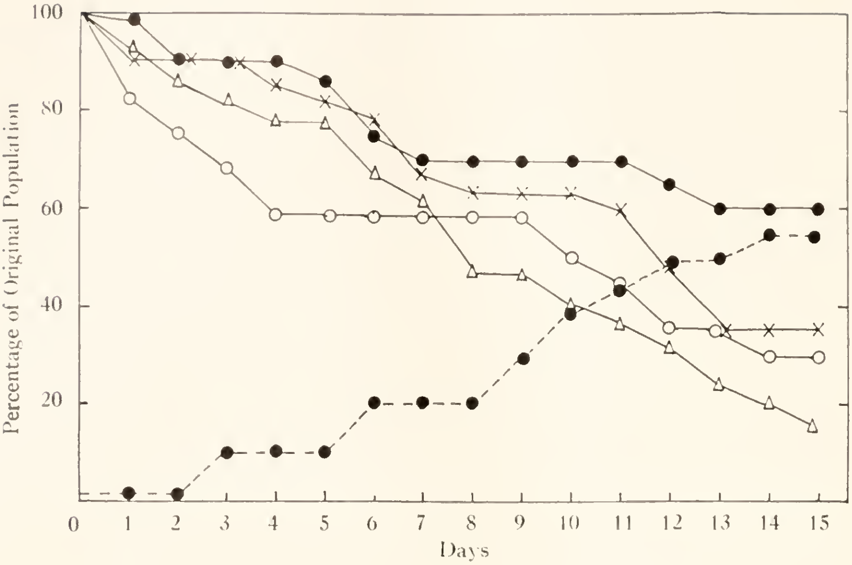
(2) Bacteria, even in high concentration, are not an adequate food for *Calanus*.

SEPARATION OF MICROÖRGANISMS INTO VARIOUS SIZES

Since bacteria were found to be unimportant as a source of food for *Calanus*, an attempt was next made to determine the size range of the organisms actually used as food by this copepod among the various other types of nannoplankton. For this purpose the animals were placed in harbor water which had been passed through a graded series of filters as indicated in Table II.

Duplicate groups "*a*" and "*b*" were first set up in each series with twenty animals each. Some unknown factor, probably a contamination of the harbor water, caused a large number of deaths on the second and third days of the experiment. Consequently the copepods from each pair were united in one container, and a new group "*c*" started in each series. The toxic agent, however, probably influenced subsequent results, for the death rates in combined "*a*" and "*b*" (hereafter called "*ab*") are in every case higher than those in the corresponding "*c*" groups. Excluding the first three days, group "*ab*" ran for fifteen days and group "*c*" for eleven days. The animals, which were kept in covered glass dishes set in a water bath at 15° C., were transferred daily to clean containers of freshly prepared sea water. Samples of water were centrifuged from time to time, and microscopical examinations of the organisms thrown down were made.

In going from untreated water to water passed through finer and finer filters one finds (Table II and Fig. 2) a consistent decrease in the ability of *Calanus* not only to moult successfully but also to survive. This is especially true if adults are disregarded, since they survived equally well under all conditions of these experiments—probably because they have stopped growing and are no longer dependent upon a regular food supply. It might be concluded from these results that *Calanus* lives best on the larger planktonic forms—diatoms, peridinians etc.—which were found abundantly in the harbor water, and which were held back by the paper filters. Another interpretation is possible, however. Microscopic examination of centrifuged samples of the paper-filtered water revealed numbers of minute flagellated organisms, but there were even more of these in similarly centrifuged samples of unfiltered water. Evidently many of the smaller organisms were caught in the meshes of the filter of each grade along with the larger ones with the result that a purely qualitative separation according to size was not attained. Therefore, it is impossible to judge from these results whether the differences between the welfare of the copepods in the several experiments were due to quantitative differences in the supply of flagellates and other small organisms, or to qualitative differences in the types of organisms available. Since bacteria were abundant in all containers (Table II), it is again clear that they are not an adequate food for *Calanus*.



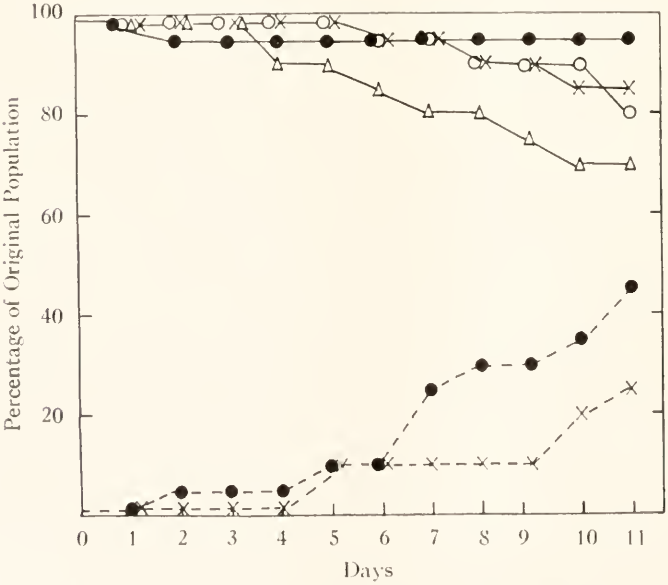


FIG. 2*a*. Series *ab*. Survival and moults of *Calanus finmarchicus* in water passed through different types of filters. Percentage of original population surviving shown by solid lines; percentage of original population moulting shown by broken lines.

Solid circles, unfiltered; crosses, Whatman 12; open circles, Whatman 40; triangles, Berkefeld W.

FIG. 2*b*. Same for series *c*.

Table II

Test as food for Calanus of microörganisms separated into various sizes.
Roman numerals refer to copepodid stages.

Series No.	Filter used	Population at start	Number of moults	Died during moult	Died, not during moult	Population at end	Bacteria per cc. on third day
1 *ab*	Untreated	5 IV	1	2	2	0	50,000
		12 V	10	0	2	1	
		3 VI	0	0	2	11*	
1 *c*		4 IV	4	0	0	0	
		14 V	5	0	1	12	
		1 VI	0	0	0	6	
2 *ab*	Whatman No. 12	2 IV	0	1	1	0	250,000
		15 V	0	6	5	4	
		5 VI	0	0	1	4	
2 *c*		5 IV	4	0	1	0	
		12 V	1	0	2	13	
		2 VI	0	0	0	3	
3 *ab*	Whatman No. 40	4 IV	0	1	0	0	560,000
		15 V	0	0	10	5	
3 *c*		4 IV	1**	1	1	1	
		12 V	0	1	1	11	
		4 VI	0	0	0	4	
4 *ab*	Berkefeld W	5 IV	0	1	4	0	400,000
		21 V	0	0	18	3	
		2 VI	0	0	0	2	
4 *c*		16 V	0	0	6	10	
		4 VI	0	0	0	4	

* Includes two males. All other Stage VI recorded are females.

** This moult occurred on first day, and was probably started when animal was captured.

THE NUTRITIONAL REQUIREMENTS AND THE FILTERING RATE OF CALANUS

Our search for the organisms which actually do serve as food for *Calanus* would be considerably narrowed if we could show that certain types are so sparsely represented in the sea that a copepod could not take in a sufficient number of them during the day to meet its nutritional requirements. The daily food requirements of a stage V *Calanus* during the summer have been calculated by Marshall, Nicholls, and Orr

(1935) on the basis of the oxygen consumption of a large number of individuals, as 0.013 mg. of carbohydrate or 0.005 mg. of fat. To obtain an approximate idea of the volume of water which a stage V *Calanus* would be required to filter to obtain this amount of nutriment, we have estimated the food content of the different elements of the phytoplankton and nannoplankton per unit volume of sea water at a station occupied on July 5, 1935, and located 7 miles south of the entrance to Vineyard Sound—a locality where *Calanus* occurred abundantly throughout the summer.

TABLE III

Daily filtering requirement. Volume of water containing sufficient organisms of certain types to supply the daily nutritive needs of a stage V *Calanus finmarchicus.*

Organisms	Number of cells	Calculated weight	Nutritive equivalent carbohydrate	Daily filtering requirement
	per cc.	*mg./cc.*	*mg./cc.*	*cc.*
Diatoms and peridinians V.S.	100	1.25×10^{-3}	1.4×10^{-4}	93
"Flagellates" V.S.	800	2.3×10^{-4}	4×10^{-5}	325
Bacteria V.S.	20,000	7.8×10^{-6}	1.3×10^{-6}	10,000
Bacteria	1,000	3.9×10^{-7}	6.5×10^{-8}	210,000
Bacteria	1,500,000	5.9×10^{-4}	9.8×10^{-5}	140

Notes: Composition of organisms assumed as follows:

	Percentage			
	Water and inert	Protein	Fat	Carbohydrate
Diatoms and peridinians	90	4	1	5
"Flagellates"	84	12	0.5	3.5
Bacteria	84	12	0.5	3.5

V.S.—Data based on quantitative analysis of water samples taken at the Vineyard Sound whistle buoy July 5, 1935.

If all three types of organisms served as food, the daily filtering requirement will be 72 cc.

The mass per cc. of diatoms, peridinians, and "flagellates" has been estimated from cell measurements on preserved material made by Miss Lois Lillick. The density of the living cells has been taken as equal to that of sea water. The mass of bacteria per cc. has been calculated from plate counts made by Dr. Margaret Hotchkiss. Each organism has been considered as a cylinder 0.5μ in diameter and 2μ long with the density of sea water. The analyses of Brandt (1898) were used as a basis for the assumed composition of diatoms and peridinians; those of Buchanan and Fulmer (1928) were used for the bacteria. We have found no published analyses of flagellate composition, and have considered it as similar to that of bacteria. Since these analyses were based upon mixed plankton samples, the values assumed and the results obtained (Table III) must be regarded as rough approximations only. The volume of water has been calculated which would have to be filtered if the copepod lived exclusively on one type of organism in the concentration in which it existed at the station considered.

Up to the present time no measurements have been made to our knowledge of the volume of water *Calanus* can effectively filter per day.[3] As measured from the drawings of Cannon (1928), the filtering surface of the appendages has an area of 0.12 sq. mm. To filter in 24 hours the minimum of 72 cc. of water (Table III) an average rate of flow through the filter of 6.9 mm. per second would be required. Whether this is a reasonable value in view of the fact that the appendages were observed by Cannon to make 10 strokes per second is a matter for speculation.

A direct measurement of the filtering rate was attempted in some entirely preliminary experiments in which *Calanus* was allowed to " feed " for a certain number of hours in a suspension of carmine particles, the concentration of which was determined before and after the feeding period. The suspension was made up in sea water and filtered through absorbent cotton. The concentration of the fine particles remaining in suspension was measured with a hemacytometer. From 10 to 15 cc. of this suspension were placed in a small glass dish and cooled to 12° C. in a water bath. Five specimens of *Calanus* were introduced on a small lifter of bolting cloth. The amount of water added with the animals caused no appreciable dilution of the carmine suspension. At intervals the concentration of carmine particles remaining in suspension was determined, and an examination made for fecal pellets containing carmine. Such pellets remain intact and do not confuse the results. A control maintained to check the effects of settling showed that carmine is unsatisfactory for use over long periods, since it settles slowly, and gradually goes into solution. For short periods, however, these effects can be neglected without seriously affecting the results. We are searching for a more satisfactory suspension with which to continue work.

The equation used to calculate the amount of water filtered over a given period of time was derived as follows. Let: $C =$ concentration of particles in the suspension; $V =$ volume of water per copepod; N

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[3] Harvey, Cooper, Lebour, and Russell (1935) report that " The copepod *Eurytemora hirundoides* has been observed by Lucas (private communication) to eat as many as 5,000 cells of the very small diatom *Nitzschia closterium* hourly, or 120,000 per day."

= number of particles per copepod; W = volume of water filtered per copepod. Then:

$$dN = -dWC,$$

$$dC = \frac{dN}{V},$$

$$-dWC = V\,dC,$$

$$W = V\ ln\ \frac{C_1}{C_2}\cdot$$

C_1 and C_2 indicate respectively the concentrations of particles at the beginning and end of a period of observation. The amount of water filtered in twenty-four hours has been calculated for one series of experiments (Table IV). The discrepancy between the value calculated after approximately six hours, and that calculated on the basis of longer periods is probably due to the fact that errors in counting have a greater effect on the results when the reduction of particle concentration is small.

TABLE IV

Filtering rate of Calanus. In each experiment 5 stage V *Calanus* were placed in 20 cc. of carmine suspension.

Experiment No.	Relative concentration carmine		Duration	Water filtered	Water filtered per day
	Start	Finish			
			hours	*cc.*	*cc.*
3	97	63	5.75	1.72	7.16
4	97	60	5.50	1.93	8.4
5	97	45	17.5	3.23	4.22
6	97	45	18.0	3.23	4.31
7	97	28	24.25	4.96	4.91
8	97	30	24.0	4.68	4.68
				Average	5.61

DISCUSSION

The results from these experiments on the filtering rate would appear to indicate that a *Calanus* cannot filter during the course of a day a sufficient volume of water to satisfy its nutritional needs if food organisms are actually as sparse as is apparently the case at the station considered. But little reliance should be placed as yet upon these entirely preliminary tests, although it is hoped that this method of investigating the filtering ability of copepods will prove valuable when perfected. The figures do show, however, that the bacteria would have to be many times more concentrated than they have ever been found in the sea in order for enough of them to exist in a volume of water which a copepod could reasonably be expected to filter in a day. This conclusion together with the results of the feeding experiments described above seems definitely to rule the bacteria out as a source of food for *Calanus*.

If copepods feed on diatoms the filtering of a smaller amount of water would be necessary, according to our results, than would be the case if " flagellates " were used provided that the number of flagellates in sea water has been correctly estimated. The total volume of the small, delicate, and easily destroyed species in the nannoplankton may turn out to be greater than supposed when adequate methods for their quantitative enumeration have been developed.

There is no doubt that the ratio of diatoms to copepods in the water is sufficiently great to meet the nutritional needs of *Calanus* if they are suitable for food and sufficiently concentrated. At the station considered only one *Calanus* was caught for each 28 liters of water theoretically strained by the net. Even though other organisms compete for the same food the potential supply would seem adequate since the rapid reproduction of the phytoplankton during periods of activity is well known.

The fact that food organisms are not evenly distributed either spatially or chronologically must also be considered. *Calanus* appears to be adapted for prolonged periods without food, since individuals in our experiments lived for nearly three weeks under starvation conditions. We have calculated the theoretical time of survival of *Calanus* assuming that metabolism continues at a normal rate under conditions of starvation, and that the total fat content is available as a reserve source of food. The average weight of fat per stage V *Calanus* in August in Loch Fyne is 0.094 mg. (Orr, 1934). Since the daily nutritional requirement of each animal is 0.005 mg. of fat (Marshall, Nicholls, and Orr, 1935), the fat reserve would last nineteen days. In early May the fat content per individual averages 0.026 mg., while the nutritional requirement is 0.003 mg. This represents a nine-day reserve. These calculations compare favorably with the results of our experiments when individual differences between animals are considered.

Before summarizing we wish to point out the gaps in our knowledge of the nutrition of *Calanus*, gaps which must be filled before a complete quantitative discussion can be given. We do not yet know the exact types of organisms utilized by *Calanus*, nor the factors influencing the proportion of each type in its diet. Data concerning the chemical composition of separate elements of the plankton are very meager, since analyses have been made on mixed catches, part of which may be unavailable for a particular species of animal. The effect of starvation upon the metabolic rate of *Calanus*, and the effect of environmental condition upon the filtering rate of this copepod are also unknown.

SUMMARY

Calanus which had been rendered practically sterile by a system of washing failed to moult when placed in sterile Berkefeld-filtered sea water but remained alive for about two weeks. The introduction of bacteria even in high concentration did not enable the copepods to moult. Rough calculations indicate that the concentration of bacteria in the sea is much too low to satisfy the nutritional needs of *Calanus*.

Calanus was found to live and moult in sea water from which the larger planktonic organisms had been removed by a paper filter, although it throve better in unfiltered water. Whether this difference was caused by qualitative or quantitative differences in the food supply has not yet been determined.

Preliminary measurements on the rate of filtration of *Calanus* gave a result less than that calculated to be necessary for the copepod to obtain sufficient food if diatoms and peridinians are the sole source of supply. Since bacteria cannot be regarded as an important source of food, the other small and little known elements of the nannoplankton should be scrutinized, as their abundance in the sea may be greater than previously supposed.

Acknowledgment

The authors are indebted to Mr. Donald Zinn, who collected the animals used in these experiments, and to Mr. Sidney Cobb, who rendered technical assistance.

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THE BUFFERING POWERS OF NATURAL AND DIALYSED HELIX POMATIA SERUM

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Redfield et al. have shown (1929) that the buffering power of reduced hemocyanin of *Limulus* is increased in the presence of salts below pH 8.0, at the expense of the buffering power between pH 8.0 and pH 9.0. The resultant increase in the effectiveness of hemocyanin as a buffer in the physiological range amounts to about 40 p.c. The present experiments indicate a similar effect of salts on the oxyhemocyanin of *Helix pomatia.* Attempts were made to obtain crystals of hemocyanin by dialysis, following Dhéré's method (1918) but in many attempts crystals were obtained twice only, and then in insufficient amount for the experiments. The hemocyanin was therefore purified by shaking the serum with chloroform, which precipitates a small amount of presumably protein material, but leaves the hemocyanin in solution, followed by dialysis of the filtrate to remove diffusible impurities. A comparison of the buffering powers of serum so treated with those of natural serum gives results qualitatively comparable with those of Redfield et al.

METHODS

Serum

Active *Helix pomatia* were used. Each batch of snails was bled after two days starvation by pericardial and heart incision. To avoid contamination with slime from the foot glands, a large aperture was made in the shell above the heart region, with a bridge of shell left between this and the shell margin. By holding a cloth over the natural shell opening, serum uncontaminated with slime was obtained. For titration curves on fresh serum, the blood was filtered and kept at 0°–2° C. until used, which was always within forty-eight hours of its drawing. The blood for the experiments on purified hemocyanin was filtered, shaken up with chloroform, again filtered, and dialysed against distilled water at 0°–2° C. until the chloride test was negative. Before carrying out the experiments, the sample was repeatedly evacuated and brought into equilibrium with air.

Chloride Determinations

The chloride determinations were carried out by the method of Van Slyke (1923) on duplicate 3 cc. samples of blood. The concentration of chloride varied from 71.5 to 85.0 milliequivalents per liter, although the samples were made up of, and therefore averaged, the sera of 20 to 200 snails. Duval (1930) found the chloride content, calculated as NaCl, of *Helix pomatia* blood when the animals were active to be 3.60–4.75 gm. per liter, i.e., 61–81 milliequivalents, values close to those found in the present experiments. During hibernation, and during dry summer weather, when the animals have retreated into their shells, higher Cl concentrations occur.

Copper Determinations

The Cu content of the samples of dialysed and untreated serum was determined by N. W. Pirie, of the Biochemical Laboratory, Cambridge. The method (Pirie, 1931) depends on the catalysis by Cu of the oxidation of cysteine in the presence of H_2O_2. The accuracy is to about 3 p.c. with the concentrations of Cu found, 0.086–0.10 mgm. per cc. in the fresh serum. Dhéré and Burdel (Burdel, 1922) found Cu concentrations of 0.065–0.075 mgm. per cc. in the blood of hibernating *Helix pomatia*.

Titration Curves

Titration of the samples was carried out as previously described (Terroux, 1931) in the cup of the glass electrode vessel. Carefully standardised HCl or NaOH was added from standard 1 cc. burettes, fitted with finely drawn out glass tips to facilitate the accurate addition of small amounts of reagent. Two 0.5 cc. portions of each sample were used for a titration curve, to one of which base was added, to the other acid.

pH Determinations

The pH of the initial sample, and the pH after each successive addition of HCl or NaOH, were determined at 21° C., after the mixture had reached equilibrium. The glass electrode apparatus described by Kerridge (1926) was used, with 3.5 N KCl in the calomel electrodes and for the liquid junction with the experimental solution. Moisture leaks over the surface of the glass electrode were obviated by working at about two degrees above room temperature, a procedure which does away with the necessity of using paraffin oil for insulation. With titration curves carried out in the cup of the electrode in this way, the same KCl contact must be used during a whole series of acid or base additions. That possible diffusion of KCl does not affect the accuracy of the determinations has been checked by the author in previous experiments (Pirie and Godwin Pinhey, 1929). Each glass electrode vessel was calibrated against a series of buffers covering the pH range under investigation, and the buffers checked by hydrogen electrode determinations. Before and after each run, the glass electrode vessel

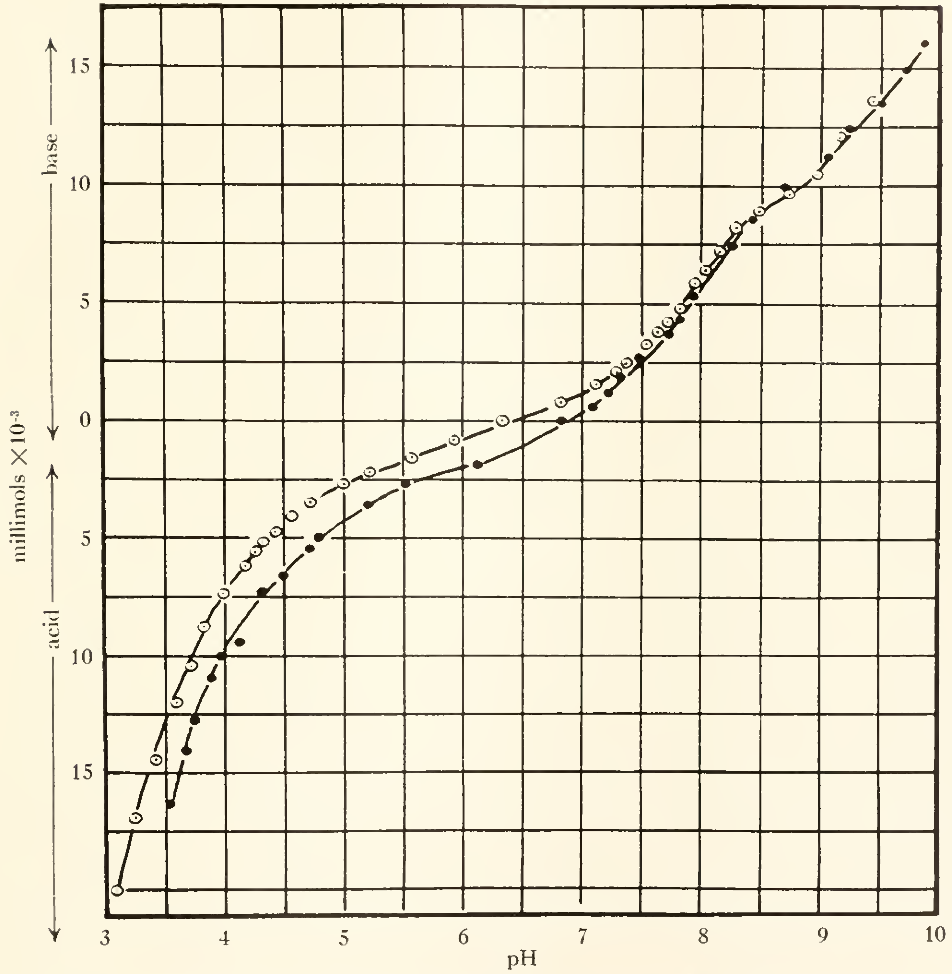

Fig. 1. Titration curves of dialysed serum; solid circle, May 20; open circle, July 23.

was checked against M/20 acid potassium phthalate, which has a pH of 3.97, and against phosphate buffer at pH 8.2. With titration curves carried out as described, each point on the curve represents a single pH determination.

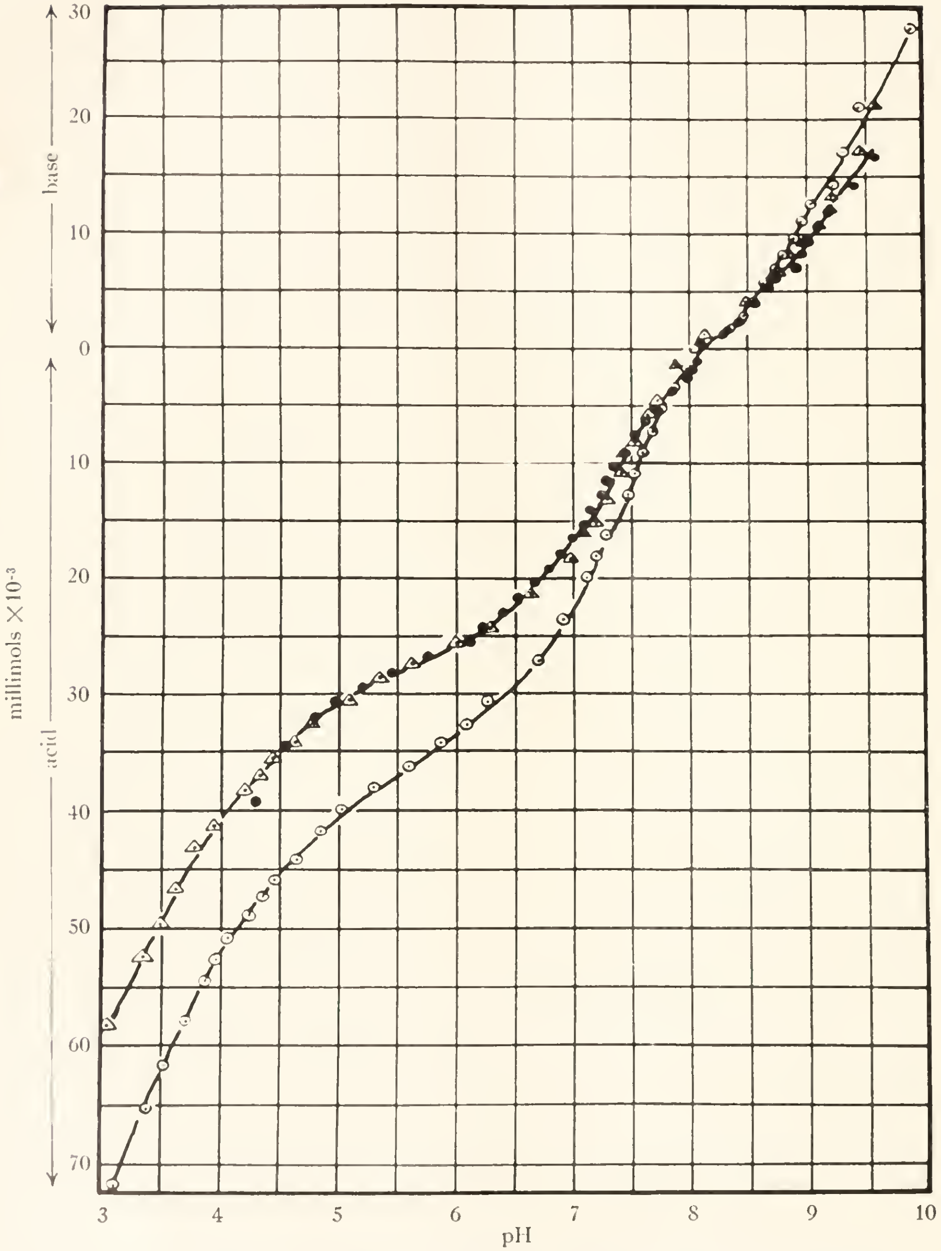

FIG. 2. Titration curves of natural serum. Open circle, July 8, 0.086 mgm. Cu per cc. sample. Triangle, July 21, 0.09 mgm. Cu per cc. sample. Solid circle, July 29, 0.10 mgm. Cu per cc. sample.

The results of the experiments are given in the figures below. For comparison, all curves are drawn on a basis of 0.1 mgm. Cu content. For the dialysed serum (Fig. 1) the buffering powers of the two samples (calculated to the same Cu and therefore the same hemocyanin content) agree fairly closely, indicating that the only substance concerned in the buffering is the oxyhemocyanin. The natural serum curves fall into two groups when plotted on the 0.1 mgm. Cu basis. The curves

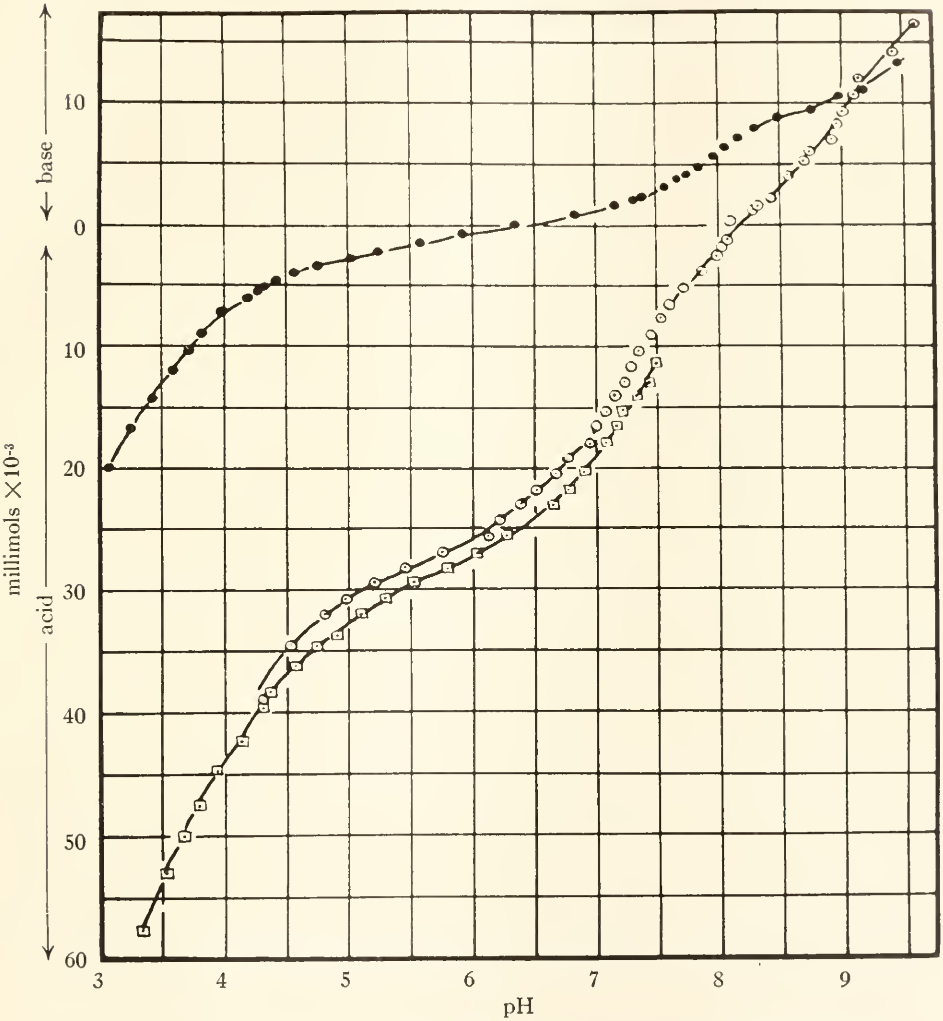

Fig. 3. Comparison of titration curves of dialysed and natural serum and test of repeatability of determinations. Solid circle, July 23, dialysed serum. Square, July 28, natural serum. Open circle, July 29, same serum as July 28.

for the samples containing 0.1 and 0.09 mgm. Cu per cc. coincide. The chloride contents were respectively 85 and 80 milliequivalents Cl per liter. The curve for the sample containing 0.086 mgm. Cu and 71.5 milliequivalents Cl apparently has a higher buffering power, as shown by the increased steepness of the titration curve (Fig. 2). This may possibly be due to a higher carbonate content, as Duval (1929) has shown that dilution of the blood in the living animal results in a lowering of the Cl concentration, but an increased CO_2 content. In Fig. 3, the titration curve of dialysed serum is compared with that of undialysed. The two lower curves in this figure are from results on the same sample of serum on two successive days to test the repeatibility of the observations.

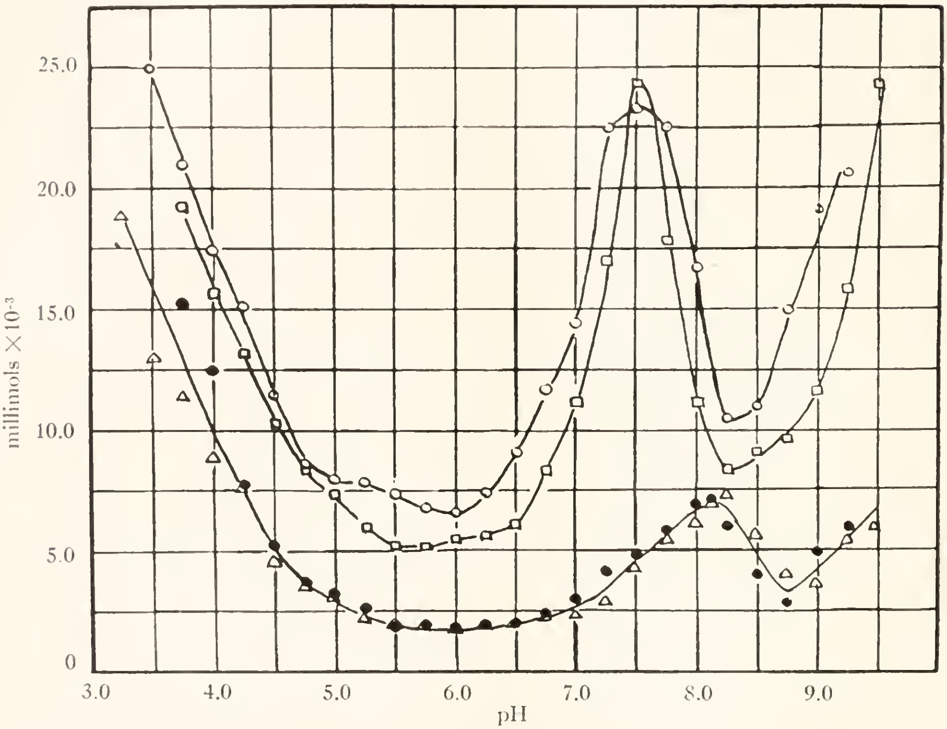

FIG. 4. Buffering powers of natural and dialysed serum. Open circle, natural serum, 0.086 mgm. Cu per cc. sample. Square, natural serum, 0.09 and 0.10 mgm. Cu per cc. sample. Solid circle, dialysed serum May 20. Triangle, dialysed serum July 23.

The buffering power of a solution is expressed by the equation

$$\beta = \frac{dB}{dpH}$$

(Van Slyke et al., 1922). For unit change in pH, β therefore is the amount of acid or base required to effect this change. The titration curves above give the buffering power of the hemocyanin containing solutions in terms of 0.1 mgm. Cu per cc. and 10^{-3} millimols acid or base. The slopes of these curves have been determined at intervals of 0.25 pH, and the results plotted in Fig. 4. The figure shows the higher buffering power of the serum containing 0.086 mgm. Cu per cc., but the correspondence of the shapes of the curves and the points of minimal and maximal buffering show that for all three samples of fresh serum the same buffering systems are involved.

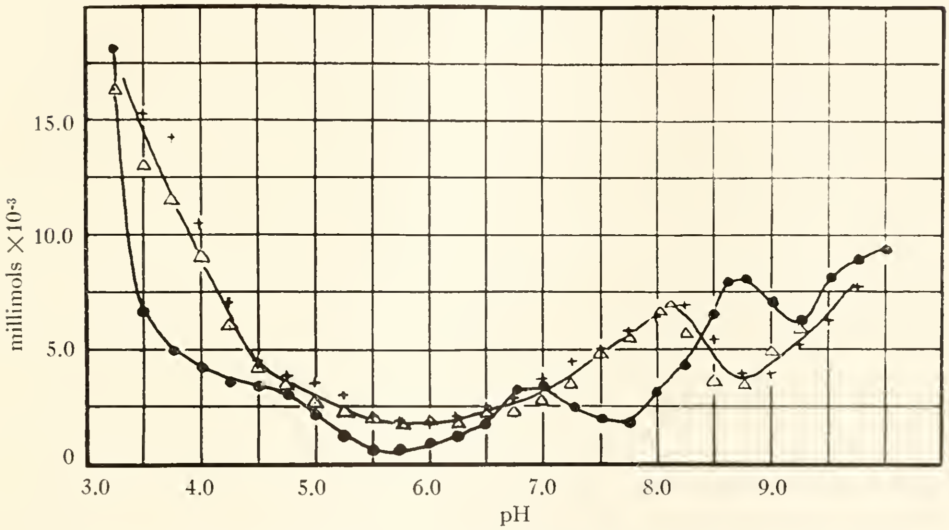

FIG. 5. Buffering power of Roche hemocyanin (represented by solid circle); of dialysed oxygenated serum May 2 (represented by cross); and of dialysed oxygenated serum July 23 (represented by triangle).

DISCUSSION

The chloroform-treated, dialysed solutions used in the experiments were not pure isoelectric oxyhemocyanin in solution, since the pH's of the two preparations were 6.82 and 6.34, and *Helix pomatia* hemocyanin has been shown to have an isoelectric point of 5.2–5.3 (Tiselius, quoted from Roche, 1931, and Stedman and Stedman, 1927). Proteins in the blood other than hemocyanin might be expected to exert a buffer effect, and would not be removed by dialysis. Cuénot (1892) found that in *Helix* blood there were no proteins precipitated by acetic, as in the case of crustacean and cephalopod blood, and further that all the protein coagulable by heat was precipitated at one temperature, 69° C. He concluded that there was present only one albuminoid, the hemocyanin.

The concentration in the solutions of buffering substances other than hemocyanin can be estimated by a comparison of the buffering powers of the solutions used with that of a solution of pure isolectric hemocyanin. Roche (1931) has published a titration curve of such a solution of hemocyanin in which the nitrogen content was 78.9 mgm. His method was the same as that used in the present experiments, in that he added either HCl or NaOH to portions of the solution to obtain his curve. The pH was determined by the hydrogen electrode, so that presumably his findings are referable to reduced hemocyanin. After redrawing his published curve on a larger scale, the slope was determined at intervals of 0.25 pH. Roche has elsewhere (1933) published analyses of crystalline *Helix pomatia* hemocyanin, and gives a table averaging his results with those of Hernler and Philippi (1930). The N content is 15.14 p.c., the Cu content 0.250 p.c. Using these figures, Roche's buffering powers and those of my samples were calculated in terms of millimols $\times 10^{-3}$ acid or base and 0.1 mgm. Cu per cc., and plotted in Fig. 5. Although the two curves do not coincide, both show a minimum in buffering power between pH 5.5 and pH 6.0, a local maximum between pH 8.0 and 9.0, followed by a second minimum between pH 8.75 and 9.25. Moreover, the total buffer values are very close for the two curves. There can accordingly be present no considerable amount of buffer substance effective in the physiological range other than hemocyanin, so that the buffering power of the purified solutions dealt with can be considered close to that of pure oxyhemocyanin. From both Roche's curve and that resulting from the above experiments it appears that buffering due to hemocyanin is at a minimum at pH 5.5–5.75, on the alkaline side of the isoelectric point.

Roche deduces from his curve that hemocyanin behaves on the alkaline side of its isoelectric point as a polyvalent acid with two pK's, one between 7.1 and 8.8, and one at about pH 10.5. The maximum efficiency of a buffer is at that pH where equal amounts of the buffer are in the forms of the free acid and its alkaline salt, that is, when the pH of the solution equals the pK of the buffer. The pK can therefore be found by a determination of the point of maximum buffering. Roche's material gives a pK at 8.6. The corresponding pK of the present material (assuming that it is due to oxyhemocyanin only) appears to be lower, at about pH 8.2. Kerridge (1926) working on *Maia* blood, found that the maximum buffering of CO_2 by reduced blood was at pH 6.39, of the same blood oxygenated, at pH 6.205. Oxygenation had decreased the maximum by 0.185 pH units, a shift that

Kerridge accounts for by the fact that oxyhemocyanin is a stronger acid than reduced hemocyanin, and so competes more efficiently with CO_2 for the available base. A similar effect of oxygenation could account for the difference between the pH of maximum buffering, or pK of my solutions and that of Roche's reduced hemocyanin.

The interpretation of the rôle of hemocyanin in the buffering of the natural blood is more complicated. Unlike *Limulus*, where the blood contains only a trace of carbonate (Redfield et al., 1929) when the CO_2 pressure is reduced to zero, *Helix* blood from active animals has been shown to contain as much as 50 vols. p.c. CO_2 when the tension of this gas is zero (Nitzescu and Cosma, 1927). The high CO_2 content has been confirmed and extended by Duval (1930), who finds that in *Helix pomatia* when active there is an increase in the blood volume, correlated with a decrease in the chloride concentration and increase in the percentage of CO_2. Duval further finds that the total ionic concentration of the blood, as measured by the depression of the freezing point, can be accounted for by the sum of the depressions due to the chlorides and carbonates present. Phosphates occur only in a concentration of 1.9–3.0 mgm. inorganic phosphorus per liter (Duval and Portier, 1928). If the concentration of the chlorides and CO_2 compounds can account for the total freezing point depression, other substances of low molecular weight present must be in quantities sufficiently minute to be negligible in considering the total buffer action of the blood. The non-protein nitrogenous compounds present cannot have any appreciable buffer action. The blood of *Helix pomatia* contains only 0.11 gm. per liter non-protein N (Delaunay, 1912), about the same concentration as in *Limulus,* in which Redfield et al. (1929) found 0.12 gm. N non-coagulable by heat per liter blood. If, as Cuénot concluded, and a comparison with Roche's results indicates, the only protein present in the blood in significant quantity is hemocyanin, the buffering action of the blood must be accounted for by the carbonates and hemocyanin present.

Figures for the CO_2 content of *Helix pomatia* blood in equilibrium with air are given by Duval. For active snails, with chloride concentrations of 3.74–4.75 gm. NaCl per liter, the CO_2 content of the blood was 47.5–61.0 vols. per cent, an average of 54.4 vols., or 0.0243 millimols per cc. Since the chloride concentration of the natural blood in the present experiments was about the same, 71.5–85.0 milliequivalents per liter, as compared with 61–81 in Duval's experiments, a calculation of the buffering due to the CO_2 based on his figures should give an approximation of the extent of buffering to be expected from the CO_2 in the present samples. The Cu content of the samples was 0.10, 0.086, 0.09 mgm. per cc., so that the curves calculated for 0.10 mgm. Cu may be taken to represent the buffering of 1 cc. blood. The pH of the freshly drawn blood was 7.8–7.9. Peters and Van Slyke (1931) give a table of the percentage of CO_2 which will be present as carbonic acid, bicarbonate, and carbonate at pH 7.8, the pH of *Helix* blood. According to their figures 2 per cent will be carbonic acid, 1 per cent carbonate, and the rest bicarbonate. In changing to a pH of 7.0, the percentage increase in carbonic acid can be calculated from the equation

$$\frac{[H_2CO_3]}{[CO_2]} = \frac{1}{1 + \dfrac{K'}{[H^+]}}$$

to be from 2 to 11 per cent, or an increase of 9 per cent. This means that 0.0022 millimols bicarbonate will be converted into carbonic acid, a conversion requiring 2.2×10^{-3} millimols acid. But the sample of serum containing 0.1 mgm. Cu per cc. required 11.25×10^{-3} millimols to effect a similar change in pH. It is evident that the bicarbonates in themselves cannot account for more than about 20 per cent of the total buffer action of the blood. Further, if the CO_2 compounds were exerting the major buffer effect, the maximum found for the blood at pH 7.5 would be inexplicable, since the maximum buffering of a bicarbonate-carbonic acid buffer is at pH 6.1.

Comparison of the buffer curves in Fig. 4 shows the greater buffering power of the untreated blood. The buffering is not only greater over the whole range investigated for solutions containing the same amount of hemocyanin as in the dialysed preparations, but the pH at which maximum buffering occurs is at 7.5 for the natural blood, at pH 8.2 in the purified solutions. The only buffer substances found in any quantity in the blood are hemocyanin and bicarbonates. The sum of the independent buffering of these two cannot account for the total buffer action of the blood. This and the shift in the pH of maximum buffering must then be attributed to alterations in the buffering power of the hemocyanin brought about in the presence of diffusible substances—of which those present in predominant concentration are the chlorides.

SUMMARY

Using the glass electrode for pH determinations titration curves of oxygenated fresh and dialysed *Helix pomatia* blood were determined.

From the slopes of these curves, buffering power was determined at intervals of 0.25 pH, and curves of the buffering powers drawn over the range pH 3.5–9.5

The fresh blood has a higher buffering power over the whole range, the difference becoming most marked at pH 7.5, where the curve for the blood shows a local maximum buffering. A similar maximum for the dialysed blood occurs at pH 8.2.

The bicarbonates present can account for about 20 per cent of the total buffering of the blood. Evidence is given that the remaining 80 per cent of the buffering is attributable to hemocyanin, and that the buffering power of the hemocyanin is augmented by the presence of salts.

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SEX INVERSION IN TEREDO NAVALIS AND ITS RELATION TO SEX RATIOS

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In two important papers on the sexual cycle in *Teredo navalis*, Coe (1933 and 1934) has undertaken, among other things, to explain the observed predominance of females over males in adult life. This excess of females over males has been reported by various investigators to be as high as 97 per cent to 99.7 per cent (Nelson, 1922). Professor Coe, on the other hand, finds himself in practical agreement with Quatrefages (1849), who recorded observation of 8.6 per cent males in *Teredo norvegicus*. From various data, direct and indirect, Coe has concluded that there are 9 per cent males in adult populations of *Teredo navalis* after the middle of the breeding season. He believes, in other words, that approximately 10 per cent of all individuals remain permanently males. These he calls "true males." The others are considered to be protandric females so that the adult population would ultimately appear to be 90 per cent females.

A reinvestigation of sex ratios in the shipworm, *Teredo navalis*, an account of which is given in this paper, has therefore been made in response to the conclusion reached by Coe (1933) that females in this and other species of shipworm greatly outnumber the males. This conclusion or generalization forms the background and foundation of his theory of protandry, as applied to this species, which, stated briefly, assumes that all young teredos develop first a male phase during which the gonads produce functional sperm. This is followed promptly by a female phase which, according to the theory as first enunciated, persists during the remainder of the life of the individual. This theory, and especially its implications, are so definitely in conflict with observations of sex ratios in *Teredo* published by the senior author of this paper (Grave, 1928) that a reinvestigation was undertaken during the summer of 1935; particular attention being given to full-grown individuals which, in accordance with Coe's theory, should ultimately be 90 per cent females.

In a later note in *Science* (1934) Coe indicates that at the beginning of the second spawning season there may be a second male phase similar to the first, but that it also is brief in duration and affects only the oldest individuals of the preceding season which have already passed through male and female phases. The reorganization of the gonads of this considerable group of young shipworms during the winter and early spring serves to increase greatly the ratio of males to females so that at the opening of the breeding season it is estimated to be four or more males to one female in the whole population. Most of these presumably transform into females again early in the second spawning season. He therefore concludes there is a great preponderance of females over males in adult life. His data show that this change from the male to the female phase takes place mainly during late May and early June so that the sex ratio is reversed at the opening of the second breeding season, the females predominating in a ratio of forty-eight males to one hundred females (Coe, Table I, 1933).

Our observations are not in opposition to Coe's conclusion that there is an early male phase but to the second stipulation that the female phase which follows persists during the entire adult life of the individual with the exception noted. This is, in fact, a necessary or fundamental postulate to account for the supposed great preponderance of females over males in adult life. As we understand his argument, this is one of the chief facts to be accounted for by any theory and constitutes the background of the theory of protandry as applied to this species, particularly in the first article (Coe, 1933). We find, on the contrary, that sex inversion continues to occur throughout the life of an individual and that in any shipworm population of adult animals there is no such significant difference in numbers of males and females. The problem at issue, therefore, is whether or not protandry is the correct term to apply to the sexual cycle of *Teredo* and whether as a consequence the adults are nearly all female or whether the sexes are more nearly equal in number.

A second purpose of our present study was an investigation of sex inversion in Mollusca in general, which has received considerable attention in recent years beginning with a paper on *Crepidula* by Gould (1917). A sufficiently extensive study of the peculiar hermaphroditism which occurs in various species of mollusk should throw light on the problem of the determination or differentiation of sex as influenced by environmental and heredity factors.

SEX RATIOS IN TEREDO

A male may be defined, for the purposes of this study, as an individual which is producing active sperm in abundance and which, for the time being, is not liberating fertilizable eggs. In addition to such males there are a few (not rare) adult individuals which are hermaphroditic in that they are carrying embryos in their brood pouches in various stages of development and which at the same time are producing active spermatozoa. In some of these, both eggs and spermatozoa intermingle in the lobules and ducts of the gonads. (See Figs. 3 and 4.) These individuals are, for the time being at least, truly hermaphroditic and provide every condition requisite to self-fertilization. A very few individuals are producing neither eggs nor spermatozoa, the gonads being empty and apparently collapsed or "spawned out." These are designated in the table as not spawning. They may be found at the height of the breeding season in July but are apparently more numerous in August. Some of these are parasitised and injured by a ciliate or a sporozoan, both of which are of common occurrence in the gonads of *Teredo*. To complete the classification, there are many true females in which few or no spermatozoa or spermatogonia are visible and true males with no ovocytes in the gonads. These so-called "true males" are far more numerous in a population of adult *Teredo* than has been supposed and apparently more abundant at the end of the breeding season than earlier.

TABLE I

Sex ratios in Teredo navalis

Date of collection	Character of individuals examined	Females	Males	Hermaphroditic	Not spawning
June 28	General population	49	11	0	5
" 29	Large specimens	23	6	1	0
" 29	General population	68	34	0	12
" 30	" "	10	7	0	0
" 28 to 30	(Totals for June)	150	58	1	17
July 1	General population	65	53	15	4
" 3	Large specimens	8	5	1	0
" 5	" "	28	27	4	0
" 6	" "	6	8	3	0
" 6	Medium large specimens	11	4	1	0
" 6	Small specimens	6	4	0	0
" 7	Large specimens	11	8	2	0
" 19	" "	9	3	0	1
" 20	" "	3	4	3	0
" 1 to 20	(Totals for July)	157	115	25	5
August 1	Large specimens	25	27	7	5
" 8	" "	19	25	4	2
" 27	" "	2	3	1	0
" 1 to 27	(Totals for August)	46	65	12	7
	Totals for entire season	353	238	27	29

(Fig. 1.) Coe estimates them at approximately 10 per cent of the population.

Table 1 contains the results of examinations of living animals made during the present summer according to the above classification. It should be noted that the column labelled " males " includes, besides true males, many whose gonads contain ovocytes in various stages of development, and varying in number from a few scattered ovocytes to a complete layer of them in the epithelial wall of the lobules of the gonad. A male, according to this classification, as stated above, is an individual which is liberating abundant active spermatozoa and no eggs. In the same way some individuals classed as females in the table have a few or numerous spermatogonia among the ovocytes but are not for the time being shedding active spermatozoa. In these individuals either the male or the female phase is predominating to the exclusion of the other.

The teredos which were examined this summer were all taken from timbers placed in the water between August 1 and August 10, 1934. Those marked " General Population " were taken from crates made of 1 × 3″ boards and were crowded and relatively small. Those marked " Large Specimens " were taken from 2 × 4″ stakes and were large individuals, having grown under good conditions. They may be considered adults ten or more months old. The point of importance is that we are dealing with adult animals of the second season and not with young animals.

The tabulated data show that males may increase in number rather than decrease during the course of a breeding season and in no sense do they decrease to the vanishing point or to 10 per cent as required by the theory of protandry. No young teredos of the present season are included in this study but only those that entered the wood last year.

Tabulations of the ratios of the sexes made in former years (1923, 1924, and 1925) from which representative data were published (Grave, 1928), show also a preponderance of females over males but not to an extent such as has been frequently reported. A high ratio of males prevails whether large individuals are selected or whether the entire population is considered. Moreover, except in June when females are relatively abundant, the ratio of the sexes remains nearly the same throughout the breeding season but with a definite increase toward maleness. The conclusion is, therefore, warranted that both in the general population and in adult individuals the sex ratio of *Teredo* at Woods Hole is not less than 30 and may be as high as 50 per cent males.

SEX INVERSION IN TEREDO

A bisexual condition of the gonads of various gasteropods and lamellibranchs has been observed by several investigators and it is generally believed that it is not a permanent condition but rather that the same individual may function as a male at one time and later become a female. This inversion of the sexes may take place several times during the lifetime of an individual as, for example, in the California oyster, *Ostrea lurida* (Coe, 1933). During the period of change of sex in *Teredo* the gonad appears hermaphroditic and sometimes contains active spermatozoa and fertilizable eggs as well as developing ovocytes and spermatogonia. This condition of true hermaphroditism has frequently been observed to obtain in *Teredo* for a short period just at the time of change from the male to the female phase and from the female to the male phase. Usually, however, one sex predominates and although both ovocytes and spermatogonia may be present, only one is maturing into functional gametes. Sections of a large number of teredos, therefore, show all gradations between maleness and femaleness and some that appear to be entirely male or entirely female. We, in fact, have found every type of gonad described by Coe and some even more convincing cases proving inversion of the sexes. These special cases are described below.

A careful study of adult individuals leads us to doubt if there is such a thing as a true male or a true female *Teredo* in the sense that it remains permanently one sex, as postulated by Coe, who agrees that sex inversion in true males may ultimately take place (Coe, 1933). It is evident from these studies that full-grown teredos show the same sex inversion that Coe has recognized in the younger animals. We believe the condition in *Teredo* is strictly comparable to that he described for the California oyster and to the snail, *Valvata tricarinata* (Furrow, 1935), rather than that of *Crepidula* in which an early male phase is apparently followed by a permanent female phase.

There is abundant evidence that a female *Teredo* may spawn two or three times and then rapidly change to a functional male. Several fully-grown individuals which were carrying both veliger larvæ and cleaving

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FIG. 1. Section of the gonad of an individual in the male phase. Spermatozoa and spermatogonia abundant. Little, if any, trace of ovocytes visible.

FIG. 2. Section of the gonad of an individual in the female phase. The small nuclei which surround the eggs belong to nurse cells. There are few if any spermatogonia in this gonad at this time.

FIG. 3. Section of an hermaphroditic gonad. It is changing from the female phase to the male phase. The walls of the lobules are composed of proliferating spermatogonia and the cavities are filled with spermatozoa and free ovocytes. This gonad is in the active male phase, and after the last of the ovocytes have passed to the exterior it will appear to be wholly male.

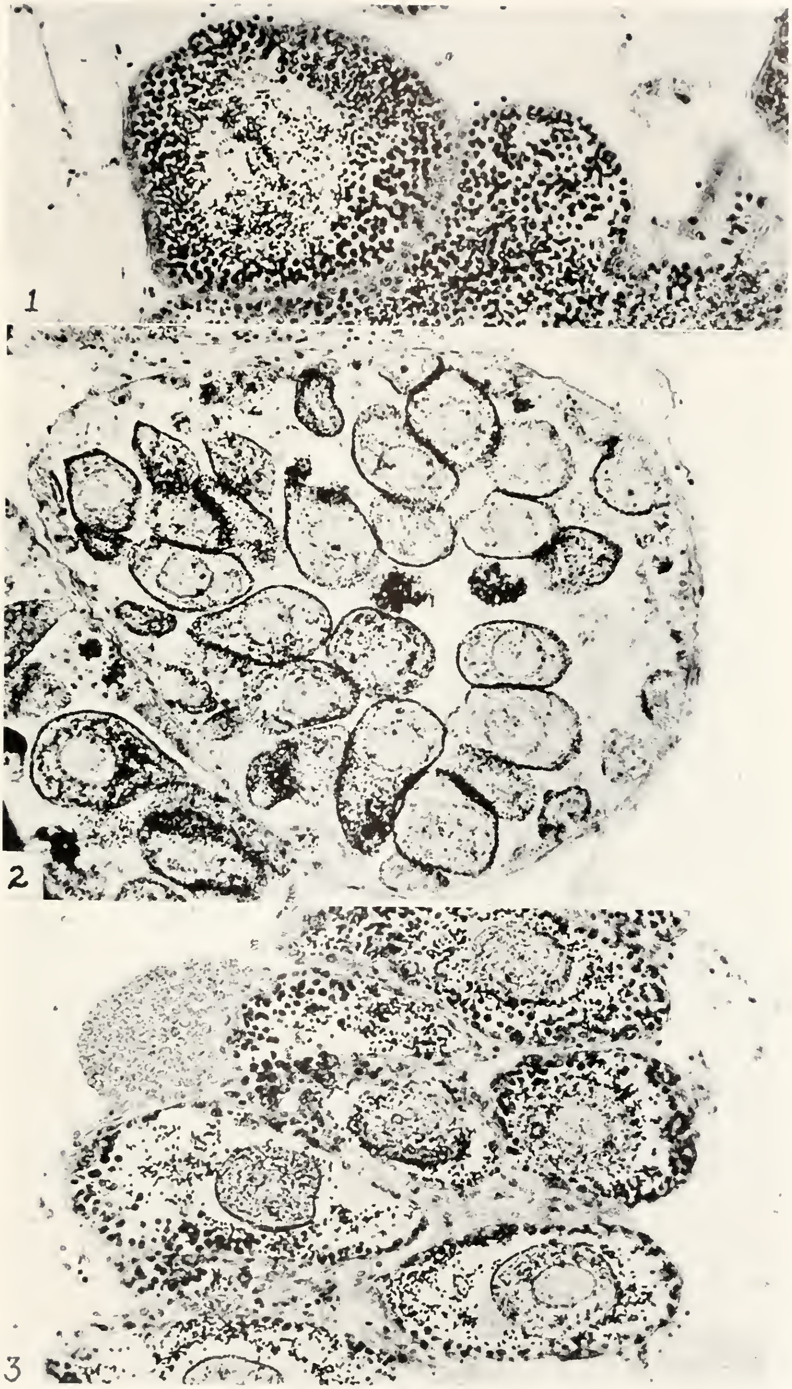

PLATE I

eggs in their brood pouches were at the same time producing active spermatozoa. The gonads of these individuals, when sectioned and stained, were seen to be functional testes with little trace of ovocytes remaining. These several examples of this type are sufficient to prove that there is sex inversion in adults from female to male. Other cases were frequently observed in which the lobules and ducts of the gonads contained free ovocytes with germinal vesicle intact while the walls of the lobule consisted of spermatogonia and spermatids. At the same time there were abundant spermatozoa free in the lumen surrounding the eggs. (Fig. 3.) That is, of course, a truly hermaphroditic condition, but since there are no large ovocytes in the wall of the lobules it is evident that the male phase is in the process of establishment from an antecedent female phase. Figure 4 shows the reverse condition in which ovulation is beginning while the lumina of the lobules of the gonad are still filled with spermatozoa. The last of the spermatogonia and spermatids are disappearing. These examples indicate that the functional change is accomplished rapidly because, in the first case, cleaving eggs in the brood pouches indicate their recent arrival there, yet the gonad had already become exclusively male. There is abundant evidence also that the hermaphroditic condition in some individuals is of short duration and that dominance of the male or female condition is the rule. Relatively few individuals are extruding both eggs and spermatozoa at the same time. There may be some truly hermaphroditic individuals but it appears more likely a temporary transitory condition of brief duration. The " spawned out " condition in which no gametes are visible in teased preparations of gonads, when not caused by parasites, is apparently a transition stage between sexual phases.

Figure 4 and possibly Fig. 5 represent types of gonads in which the male phase is coming to a close. The ovocytes in the latter are still small though abundant; Fig. 6 shows a condition in which the male phase is active in that the wall is composed largely of spermatogonia and spermatids while the lumen is filled with spermatozoa. A few scattered, half-grown ovocytes are seen in the wall which will presumably replace the spermatogonia. They probably remain quiescent during the active male phase. Figure 1 represents the male phase in which no ovocytes

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FIG. 4. Section of an hermaphroditic gonad which is changing from the male phase to the female phase. The lumen of the lobule, shown in the photograph, is filled with spermatozoa and a few dividing spermatogonia are visible between the developing ovocytes. The male phase is almost completed.

FIGS. 5 and 6 are sections of gonads in the active male phase. The lumina of the lobules are filled with spermatozoa and the walls contain dividing spermatogonia and spermatocytes. Small scattered ovocytes are visible in the wall of the gonad and these, presumably, will ultimately replace the spermatogonia and transform the gonad into an ovary.

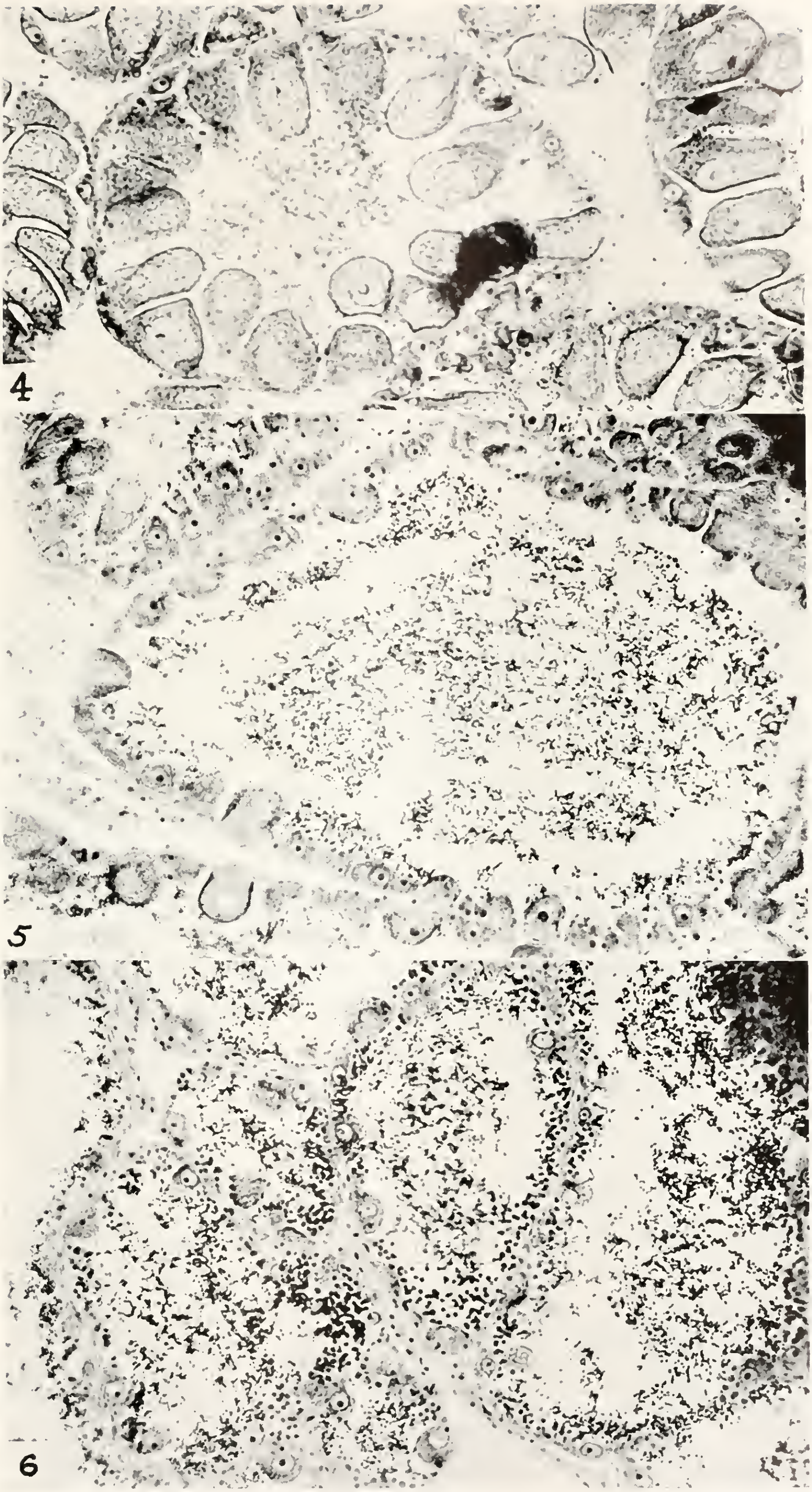

Plate II

appear; Fig. 2 represents the female phase in which few or no spermatogonia can be certainly identified but presumably potential spermatogonia exist. They are, in fact, often clearly visible between the ovocytes of so-called females. It should be explained that the small nuclei which surround the eggs are not spermatogonia but belong to a chorion of nurse cells. They are much smaller than those of spermatogonia and apparently have no significance for this study.

Discussion of the Entire Life Cycle

We have not studied the very young teredos sufficiently to have a complete knowledge of all the details of this period of development. It appears, however, both from extended study of living animals and sections that an early male phase is dominant and characteristic as reported by Coe (1933), but this phase is of short duration. From the fifth to the sixth week after metamorphosis most individuals are in the active male phase but by the end of six weeks or two months many have transformed into functional females and have extruded considerable quantities of eggs as shown by the presence of embryos in the brood pouches. Although the male phase is normally of short duration and easily overlooked, there is reason to believe that some individuals become females without passing through the preliminary male phase. This applies particularly to those not very numerous individuals that extrude fertilizable eggs at six weeks of age and are therefore among the first to spawn.

It is apparent from our study of later stages that the female phase is of considerable duration. It normally covers a period of two or three months and includes two or three successive spawnings at monthly intervals. This female phase is in turn frequently followed by a second male phase which apparently persists to the end of the life of the individual. There is no apparent reason why there should not be a second female phase following upon the second male phase except that the duration of life of this species is from 12 to 15 months, which means that there are two spawning seasons at most. The noticeable increase in the ratio of males to females toward the end of adult life as shown by Table I, indicates that this sex inversion from female to male is taking place most actively from the middle to the end of the second breeding season (July and August). The sexual life cycle of these individuals would therefore be represented by: male-female-male. It is known from previous studies of *Teredo* (Grave, 1928) that young individuals which develop from eggs spawned late in the breeding season do not reach sexual maturity until the following summer. These individuals have a single breeding season and their life cycle is represented by male-female.

The sexual cycle of individuals which become females without a preliminary male phase is not known because of the difficulty of distinguishing them from others of the population.

We have not had opportunity to verify Coe's evidence that individuals which pass through both a male and a female phase during the first season may become functional males at the opening of the second breeding season. (Coe, 1934.) These are no doubt among the large group that changes from male to female early in June and thus effectively reverses the sex ratio from greatly predominant male to almost equally predominant female within a period of one month. However, we have observed that sex inversion continues though in smaller numbers into July, and except for this group described by Coe (1934) the transformation to the second female phase takes place most actively in late June and July. Individuals which behave thus are presumably among the oldest of the over-wintering population and are one year old at the opening of the second breeding season. These favored individuals therefore have two breeding seasons. In this case, since we have shown that sex inversion is not confined to young individuals but takes place also in the adult animals, the sexual life cycle of this group whether inversion happens early in the second breeding season or later, should be represented by male-female-male-female.

On the factual side we have shown sexual inversion is taking place in fully grown *Teredo* during July and August both from female to male and from male to female. *Teredo navalis* therefore resembles the California oyster in its sexual life cycle rather than *Crepidula,* which has an early more or less persistent male phase which is followed by a permanent female phase. It is therefore properly called protandric whereas *Teredo* usually has one or more additional transformations to complete its sexual cycle.

Discussion and Conclusions

I

It may be observed that there is little difference between Professor Coe's data and ours except for the last two months of the life history of *Teredo.* This part of the life history deals with data and observations for July and August: of the second breeding season. What is the explanation of the final difference of interpretation?

In analysing Coe's data in support of the belief that the adult population is 10 per cent male after the middle of the breeding season, it should be pointed out that he draws his conclusion somewhat indirectly. Thus in one case he analyses a population collected early in the summer and shows by a study of sections that it contains 10 per cent true males. The others are in the female phase or are clearly protandric females which should become functional females before the end of the breeding season, leaving 10 per cent permanent males (see Coe, 1933, p. 196). In another place (at the bottom of page 296) he used a group of 202 young teredos collected August 18–21. He shows that this group contains 18 males, which confirms the estimated ratio of 9 or 10 males to 100 females. Again on page 298, he uses a group of 220 young teredos for the same purpose. It would appear that these young animals of the current breeding season could not be confidently used in proving the existence of a certain sex ratio in adults. It is a difficult matter, it is true, to get reliable data on normal sex ratios at the end of the breeding season because so large a part of the population is dying of old age. It is evident, therefore, that the basis for the assumption that there are only 10 per cent males in adult populations of *Teredo* after the middle of the second breeding season rests upon indirect evidence which fails to take into account the continuation of sex inversion from female to male during this period as well as earlier. All of Coe's actual counts of adult animals collected during the second breeding season show not less than 30 or 35 per cent males, some of which are hermaphroditic or protandric females in the male phase of development. This group remains part of the population throughout the breeding season and is properly listed among the males for the reason that it consists of functional males producing active spermatozoa and no eggs. They frequently are devoid of distinguishable ovocytes.[1]

II

In conclusion, emphasis is given to two points which constitute the contribution of this paper to the study of sex inversion in Mollusca. First, males are without doubt much more abundant in *Teredo* than a survey of the literature indicates. They constitute from 30 to 50 per cent of adults and an even larger proportion of the young. Second, there is inversion of the sexes throughout the life of the *Teredo*. It is not confined to the very young individuals or to the opening of the second breeding season, as Coe has maintained in his papers on *Teredo*. The condition in *Teredo* is therefore strictly comparable to that of the gonads of the California oyster, *Ostrea lurida,* as described by Coe. Although the life of *Teredo* is short, covering not more than one and a half years (usually 12 to 14 months), the female after spawning two or three times at monthly intervals reverts rapidly to the male phase and produces abundant active spermatozoa. This is as much a characteristic of fully-grown teredos as of any other stage of their life history.

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[1] The abundant males which appear after the middle of August, as Coe points out, are young animals of the current season between 2 and 4 cm. in length, whereas adult animals grown under favorable conditions reach a length between 20 and 30 cm. In any case, there is no necessity for confusing them. The young teredos do not appear in the population as breeding animals until the middle of August and are easily distinguished from the older ones. Our work has dealt only with adults.

As to the cause of this inversion of the sexes, we have no theory to offer, but it seems to be due to genetic causes rather than to environmental influences.

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[2] Coe gives a more complete bibliography and review of the literature.

RELATION BETWEEN MATURATION DIVISION AND CLEAVAGE IN ARTIFICIALLY ACTIVATED EGGS OF URECHIS UNICINCTUS (VON DRASCHE)

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Recently Tyler (1931*a*, 1931*b*, 1932*a*, 1932*b*) has reported interesting studies on the relation between the polar bodies and cleavage in the parthenogenesis of *Urechis caupo.* He observed that only the eggs which were incompletely activated and did not protrude polar bodies divided and formed embryos, when unfertilized eggs were treated with diluted sea water. The chromosomes seen at the first cleavage in such eggs took the appearance of tetrad and numbered haploid. He obtained high percentages of cleavage by suppressing the protrusion of polar bodies with double treatments of diluted sea water and thought that the chromosomes were divided into two groups first and then formed the cleavage nucleus, fusing with each other in this case, as judged from the cytological observation on the preparations.

The process of parthenogenesis in *Urechis unicinctus* manifested by our experiments differs remarkably from that in *Urechis caupo* and varies somewhat from those in *Thalassema* (Lefevre, 1907), *Chætopterus* (Allyn, 1912), *Amphitrite* (Scott, 1906) and *Cumingia* (Morris, 1917). We will discuss those points briefly.

Urechis unicinctus (von Drasche) is found abundantly at the seashore in the neighbourhood of our university and matures in winter. Experiments were done at room temperature, between 9°–21° C. The sea water used was previously heated to about 80° to sterilize sperms and was then left to be cooled to room temperature. It was aerated by stirring before using. When taking out the eggs from the mother animal, the body surface of the worm was washed with fresh water, and the segmental organs, which are filled with eggs, were also thoroughly washed with sperm-free sea water. Accordingly no eggs were activated by sperms in the control of the experiments of artificial parthenogenesis. As much care as possible was taken not to stimulate mechanically the unfertilized eggs in handling. Observations were made on living and fixed materials. Among the latter, besides permanent total and section preparations, temporary preparations stained by aceto-carmine were used to determine the general behavior of the nucleus. Bouin's and Navashin's solutions were used as fixing reagents for total preparations and beside these Champy's solution was used for material to be sectioned. Before sectioning, the eggs were first attached with albumen to pieces of the thin sheath of toad liver or petals of *Chrysanthemum*, then dehydrated and imbedded in paraffin. The sections were cut at 10–25 μ. Heidenhain's iron hæmatoxylin was used for staining, and, in the case of the materials fixed by Champy's solution, they were bleached with H_2O_2; the affinity to dyes was intensified by Chura's method before staining.

The unfertilized eggs of *Urechis unicinctus* can be caused to develop artificially to various degrees by mechanical, physical, and chemical stimuli. When the eggs are put into sea water in a test-tube together with small pieces of wire net or cotton cloth and shaken lightly for 1–2 minutes, most of them are activated and finish maturation division. The maturation division is hardly distinguishable from that of fertilized eggs, except for a few eggs which show cytolysis. About 1 per cent of them cleave. Several of those which do not divide swim about, becoming ciliated larvæ later. If unfertilized eggs are exposed to warm sea water of about 31.5° C. for 10 to 30 minutes, some of them are activated, then cleave and become larvæ. Exposure to hypertonic sea water made by adding NaCl or KCl to normal sea water, causes almost all of the eggs to be activated. Some of them cleave and move about as swimming larvæ. The hypotonic sea water made by dilution with distilled water is also another agent which induces parthenogenesis. The best results were obtained by the use of ammoniacal sea water as a chemical reagent for stimulation. When exposed to a mixture of 5 cc. of 0.1 N NH_4OH and 100 cc. of normal sea water for 1 minute, most of the eggs are activated and finish maturation division. They never cleave as the result of such short treatment, but in the case of exposure for 30–40 minutes, 98–99 per cent show cleavage in the most favorable instances. Alkaline sea waters made by addition of NaOH or KOH are also effective reagents following treatment with NH_4OH sea water. Unfertilized eggs are also activated by KCN solution and several of them cleave once or twice. In the present studies we have chiefly used NH_4OH sea water as the stimulative agent for artificial parthenogenesis.

By examination of the relation between polar body protrusion and cleavage in living materials, though polar body protrusion is hindered by the stimulative agents to a certain degree, it is clear, as indicated in Table I, that there is no distinct correlation between it and cleavage, as was shown in the case of *Urechis caupo*. In the cases with one or two polar bodies, it can not be claimed that they are of the same nature in each egg. When one polar body exists, it may be the first in some cases and the second in the others. Eggs with two polar bodies are classified into three groups, namely (1) those with a first and a second polar body, (2) those with two second polar bodies without any first one, and (3) those with two derived from the first polar body, with no second one. Eggs with three polar bodies resemble the fertilized eggs but such eggs are produced very rarely.

In order to clarify the correlation between the nature of the nucleus and the cleavage, we tried the following treatments with NH_4OH solution on the eggs. In the first place, the unfertilized eggs were made to protrude polar bodies, by stimulating them only to an extent in which maturation divisions were finished, but cell division was not induced. Then the capacities of those eggs to induce cleavage were examined by the second treatment. Results of such double treatments are shown in Table II.

TABLE I

Polar body formation and percentage of cleavage

Exp. No.	Room temperature	Agent	Length of exposure	Number of polar bodies			Percentage of cleavage
				0	1	2 or 3	
	°*C.*		*min.*	*per cent*	*per cent*	*per cent*	*per cent*
22*B*	15	$\frac{1}{10}$N NH_4OH, 5 cc. + sea-water, 100 cc.	45	12	9	9	99
26*B*	14	"	30	4	2	94	80
29*E*	20	"	30	2	48	50	89

Cleavages which resulted from the above experiments become very irregular as compared with those from single treatments. The blastulæ formed by the cell divisions are composed of cells of various sizes and of abnormal forms.

It is maintained by Tyler (1932*a*) and many other workers that the spindle of the maturation division develops and becomes the cleavage spindle which induces the first cell division. Lefevre (1907) believed that when the first polar body is not formed in the eggs of *Thalassema,* two resting nuclei are produced abnormally and in most cases these two soon fuse to form the cleavage nucleus. Morris (1917) observed almost similar phenomena in the eggs of *Cumingia,* and Allyn (1912) thought that such phenomena may occur also in the eggs of *Chætopterus.*

Scott (1906) observed in *Amphitrite* that the dyads of chromosomes left in the egg as the result of first maturation division retreat to the center.

In the eggs of *Urechis unicinctus* various abnormalities take place in the maturation division and the protrusion of polar bodies is hindered as is shown in Table I, but transition of the spindle of first or second maturation division to the cleavage spindle is never seen. The maturation divisions induced by various artificial stimuli are almost alike in all eggs (irrespective of the protrusion or suppression of polar bodies) with regard to the structure of the tetrads, the uniformity of their number and the facts that they become monads by successive two divisions without any resting stage and that the resting nucleus is formed after the second division. The numbers of monads left in the matured eggs vary depending on the number and nature of the polar bodies protruded. In the cases in which two polar bodies were protruded regularly as in fertilized eggs, monads of haploid number were left; when a second polar body was not formed, monads of diploid number left; when two second polar bodies were protruded originating from two groups of dyads placed in the egg on account of lack of first polar body formation, monads of diploid number were left; when only one second polar body was formed from one of the two groups of dyads above mentioned, monads of triploid number were left (Fig. 1); by the eggs with no polar body, monads of tetraploid number were left in the egg. When the distribution of the chromosomes on the spindle

TABLE II

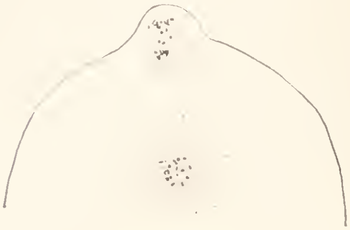
Percentage of cleavage by second treatment

Length of exposure of second treatment	Stage of second treatment	
	Immediately after extrusion of first polar body	Immediately after extrusion of second polar body
min.	*per cent*	*per cent*
5	10	0
10	12	4
20	23	8
30	31	37
40	40	25

Exp. No. 36*A*. Room temperature 16° C. Agent of first treatment 1/10 N NH_4OH solution 2.5 cc. + sea water 100 cc. Time-length of first treatment, 8 minutes. Agent of second treatment, 1/10 N NH_4OH 5 cc. + sea water 100 cc.

Upon being returned to normal sea water after the first treatment, the formation of the first polar body was 100 per cent after 42 minutes and of the second one 100 per cent after 1 hour and 12 minutes, but no eggs cleaved later, when the eggs were not treated again.

at maturation division is irregular, the number of the chromosomes left in the egg becomes irregular in most cases (Fig. 2). There is one resting nucleus composed of monads in the eggs which have completed regular maturation division, as in fertilized eggs, but there are one to four in other cases. Accordingly, it is almost impossible to infer the number of the chromosomes left in the matured eggs and the nature of the resting nucleus merely by the number of the polar bodies protruded.



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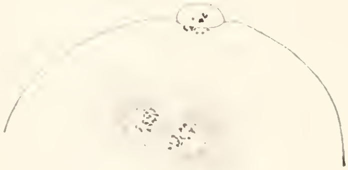

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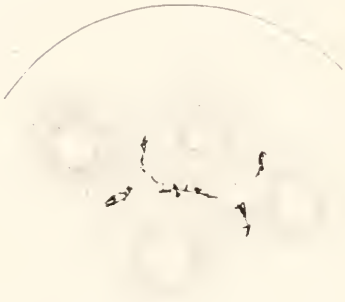

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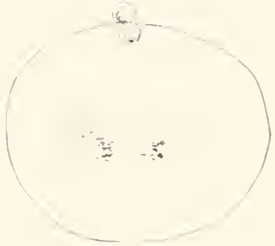

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FIG. 1. Abnormal second maturation division in which only one second polar body was formed from one of the two groups of dyads. Another group of dyads divided deep in the egg simultaneously, but in this figure only one pole of it is shown.

FIG. 2. Irregular distribution of chromosomes at maturation division. Chromosomes seen just beneath the first polar body are those which are left in the egg at the first maturation division. The second maturation division took place deep in the egg.

FIG. 3. Tetraster produced at the time of first cleavage, when more than two nuclei exist and asters which have appeared in contact with the membrane of each nucleus connected each other. Chromosomes arranged on the equatorial plate are quite abnormal and irregular.

FIG. 4. Amphiaster produced at the time of first cleavage in the egg which was supposed to have finished maturation divisions similar to the fertilized egg. The aster and spindle are small in size and irregular as compared with normal ones.

The asters which appear at the first cleavage are always placed in contact with the nuclear membrane and the cytaster is never seen.

There are sometimes two asters, as in fertilized eggs, and these form an amphiaster; but besides the amphiaster, polycentric figures such as triaster or tetraster are found. Amphiasters are mostly produced in the eggs with one nucleus and when more than two nuclei exist, asters which have appeared in contact with the membrane of each nucleus connect with one another and show a polycentric figure (Fig. 3). In any case, the form and number of the chromosomes which have appeared are very irregular and indefinite. Even when an amphiaster is produced in the egg which is supposed to have finished maturation divisions similar to the fertilized egg, the chromosomes take on irregular granular, rod-like or thread-shaped forms. Their number is recognized as about haploid (17) in some cases but often exceeds it. The aster and spindle are generally small in size and irregular. At the anaphase almost equal numbers of chromosomes move to both poles (Fig. 4). When multipolar mitosis takes place, not only the numbers of chromosomes are indefinite but also their shapes are irregular. Of course at the metaphase extremely irregular division of chromosomes results. In any case eggs usually form two blastomeres of about equal size by the division of the cytoplasm but sometimes show irregular division or no cytoplasmic division.

Other eggs produce monasters at the first cleavage. Only the chromosomes divide but the division of the nucleus is not accompanied by that of the cytoplasm, though they invariably have complete maturation divisions almost similar to those of fertilized eggs and protrude both polar bodies. Monasters which appear at this time are roughly classified into two categories by the position, structure and size of the aster and the form and number of the chromosomes. (1) Astral rays are short and radiate surrounding the groups of chromosomes. Chromosomes are crooked and thread-like, as shown at the first cleavage of fertilized eggs, and are haploid in number. (2) Astral rays develop especially in one direction and irregular chromosomes of haploid or indefinite number are scattered widely on the astral rays. The relations between treatment, maturation division, and first cleavage are summarized as follows. Weak treatments cause the maturation divisions to complete in a manner similar to that in fertilized eggs and produce a monaster at the stage comparable to the first cleavage. Moreover, the shape of chromosomes which appear at this stage resembles those at first cleavage, and their number is about haploid. Agents of strength adequate to induce cleavage often interfere with the maturation division, produce various abnormalities, and also always injure the chromosomes in the first cleavage. The achromatic figures appearing at the first cleavage are monaster, amphiaster, triaster, or tetraster and in the cases other than those with the monaster, chromosomal division is generally followed by cytoplasmic division.

In and after second cleavage the arrangement of blastomeres becomes very irregular, but eggs which have repeated divisions develop to blastulæ and few of them transform into gastrulæ and some trochophores. With the repetition of divisions, the shapes of chromosomes become constant in each cell. Among larvæ are found all stages ranging from almost normal to remarkably abnormal ones. A few of the trochophores lived for fifteen days and swam about in the water or on the bottom of the vessel with prototrochal cilia. The eggs which have repeated chromosomal division, but are not accompanied by cytoplasmic division, always show some differentiation of cytoplasm, and several of them swim about bearing cilia. In the studies of artificial parthenogenesis in Annelida and Mollusca, it is reported that the eggs of *Ophelia* (Bullot, 1904) and *Thalassema* (Lefevre, 1907) can not be differentiated without cleavage, but in many other animals such as *Amphitrite* (Scott, 1906), *Chætopterus* (Loeb, 1901; Lillie, 1902; Allyn, 1912), *Nereis* (Lillie, 1911; Just, 1915) and *Mactra* (Kostanecki, 1908, 1911) unicellular swimming larvæ are found. Such a larva of *Urechis unicinctus* has a ring of cilia quite similar to the prototroch at the equatorial position and swims with the comparatively long cilia.

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THE FOLLICULINIDÆ (INFUSORIA HETEROTRICHA) OF THE BRETON COAST

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The Infusoria of the family Folliculinidæ, which nearly all inhabit salt water, are widely distributed along the coasts. Their identification is made with difficulty by means of the tube or lorica, flask or bottle-shaped, which is the principal character employed in this case. On the Breton coast, in the bay of Concarneau, I found 15 different species. The observation of these species, in a living state or after fixation, allows one to define with precision the characteristics of species already known (2 only seemed new). It seems necessary, in spite of the excellent works of C. Dons and Kahl, to consider a revision of the group as a whole. This will be the subject of a more important study; the present paper's aim being only to give a brief description of the species found at Concarneau. As a matter of fact, I have been obliged to give particular consideration to the shape of the macronucleus. This may vary: it may be moniliform and beaded, or single, ovate or spheroidal. It seems necessary to divide the genus *Folliculina sensu stricto,* and I propose the new genus *Folliculinopsis* for the species endowed with a beaded macronucleus, the primitive generic denomination of *Folliculina* being kept for species with a single ovate macronucleus. This allows one to complete the subdivisions of the genus *Folliculina* (primitive sense) for which Dons has created several new genera.

The following descriptions are based only on the settled individuals; the swimming form has been observed for nearly all these species, but its specific characters are not sufficiently well marked to be very useful in their identification.[1]

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[1] For bibliography, refer to:

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Folliculinopsis producta Wright (Fig. 1)

This species is very common and is usually fixed on various objects (algæ, shells, etc.) in the intercotidal zone. It is easily cultivated in aquaria. The infusorian, fully expanded, is more than 1 mm. long. The tube is attached laterally for a distance of nearly 200 μ, then it bends almost at a right angle and takes the shape of a spirally marked neck (500 to 600 μ) slightly everted at its margin.

The body of the fully expanded infusorian is elongate, slender, growing very thin towards its posterior end. The two peristomal lobes are long and narrow, nearly always standing straight in the prolongation of the body. The macronucleus is made up of about 20 beads. The color of the infusorian is dark green-blue.

Folliculinopsis annulata sp. nov. (Fig. 2)

Frequently found in colonies of Bryozoa, at depths of 15 to 20 meters. The tube-length attains or surpasses 800 μ; it is composed of a flat-lying lodge, adherent, which bends upwards into a long neck, often flexuous, with annular thickenings, of which the base always shows a kind of inner collar, short and in the shape of a truncate cone.

The infusorian has never been observed more than half expanded, with the peristomal lobes elongated in parallel lines within the lorica's collar.

Folliculinopsis lignicola sp. nov. (Fig. 3)

The shape of the infusorian is very much like that of *F. producta*, but fully extended its length does not exceed 350 μ. The macronucleus consists of a dozen beads; the infusorian has a pale blue color. This small species seems to be associated with *Limnoria lignorum*, for it builds its rudimentary shell in the pitted tracheids which are cut through by the hollowed galleries of these isopods in the pine wood of submerged wrecks.

Folliculinopsis gunneri C. Dons (Fig. 4)

The blue-green, fully expanded infusorian measures almost 400 μ; the shell, lying flat, is continued by a wide, slightly upturned neck, everted at its margin, often irregular; its wall is thick and brownish. This species resembles *Folliculina aculeata* in its general aspect, but differs from it by being smaller in size and having a moniliform macronucleus of 7 to 8 beads. It has been encountered in Concarneau, not on the integument of *Limnoria*, as was observed by Dons, but on the surface of galleries hollowed by this isopod.

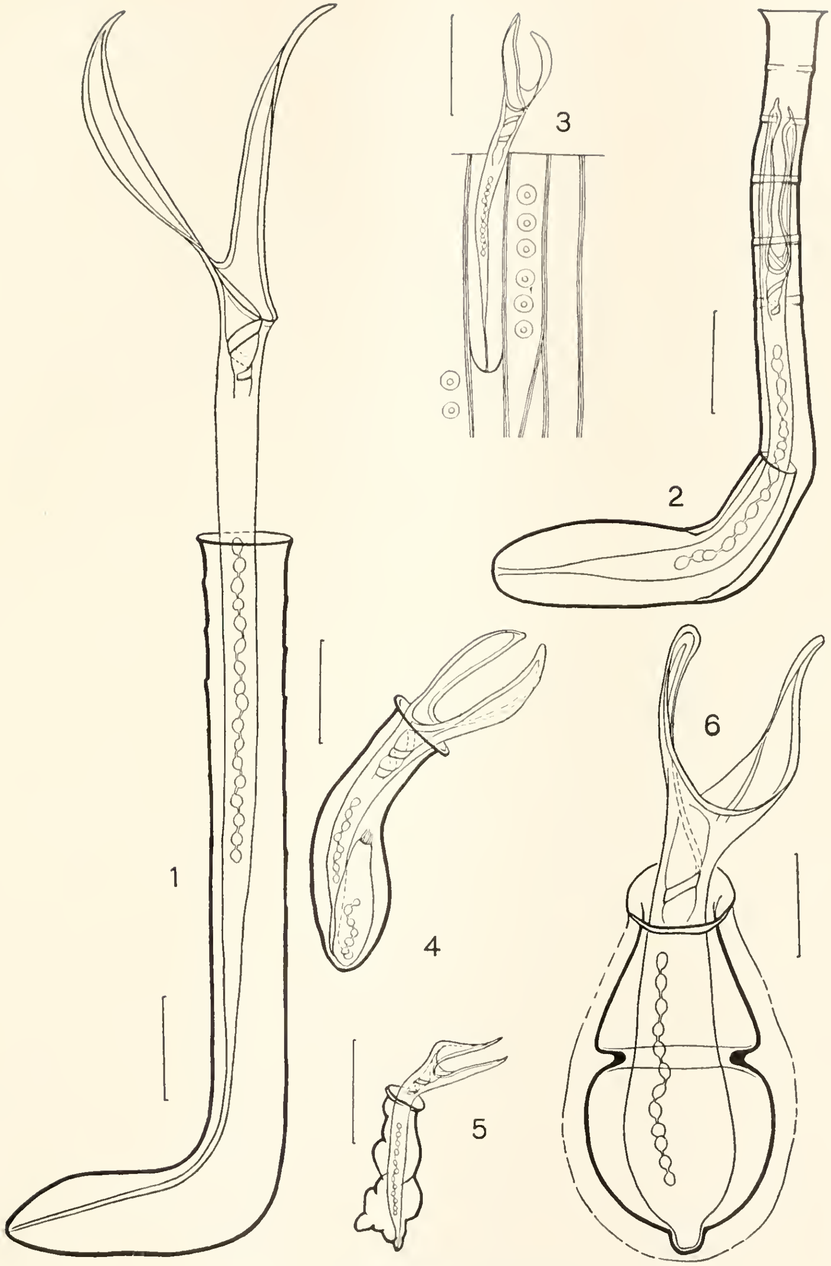

Fig. 1. *Folliculinopsis producta.*
Fig. 2. *Folliculinopsis annulata.*
Fig. 3. *Folliculinopsis lignicola.*
Fig. 4. *Folliculinopsis gunneri.*
Fig. 5. *Folliculinopsis limnoria.*
Fig. 6. *Pebrilla paguri.*
Scale indicated = 100 μ.

Folliculinopsis (*Mirofolliculina* Dons) *limnoriæ* Giard (Fig. 5)

This small species, always fixed on the last segments of *Limnoria lignorum*, is well characterized by the very irregular shape of its shell, which is 130–170 μ long. This is terminated by a very short collar, and spreads out to a large size with finger-like prolongations which may be extremely variable from one individual to another. Dons has established the genus *Mirofolliculina* for this single species; but the changing irregularity of the tube is a character of secondary importance. The expanded infusorian is 210–230 μ long; the beaded macronucleus (insufficiently observed by Giard) obliges one to rank this species within the genus *Folliculinopsis*, near to *F. gunneri*, from which it is distinguished first of all by a marked difference in size and by its specific association with *Limnoria lignorum*.

Folliculinopsis (*Pebrilla* Giard) *paguri* Giard (Fig. 6)

The genus *Pebrilla* Giard has been established for this single species of which the shell lies attached on the integument of *Eupagurus bernhardus*. This shell shows a very short and widely everted collar, a very pronounced transversal median wrinkle and a posterior stud.

The fully expanded infusorian approaches 600 μ in length, the shell 360–400 μ. I have frequently found this large and very characteristic species on the abdomen of *Eupagurus bernhardus* and of *Clibanarius misanthropus*. The macronucleus is always moniliform and shows a dozen beads.

Folliculina elegans Clap. and Lach. (Fig. 7)

This species, existing in great numbers on the Atlantic coast, attaches itself on various objects and is easily cultivated on submerged glass plates. I studied it at Concarneau and at Woods Hole. It certainly has often been described under the specific name of *ampulla;* but confusion is here impossible, as the macronucleus is single and oval, while the two folliculines described by Kahl under the names of *Ampulla* Kahl and *Möbiusi* Möbius [2] show a beaded macronucleus and are to be classified in the genus *Folliculinopsis*.

The tube consists of a horizontal lying lodge 240–270 μ in length and of a neck bending up at an angle of about 45°, slightly everted at its margin. The expanded infusorian is nearly 600 μ long, the posterior part enlarged, straightened into a long neck which precedes the peristome; both lobes run into a short sharp end.

[2] I did not observe these forms at Concarneau.

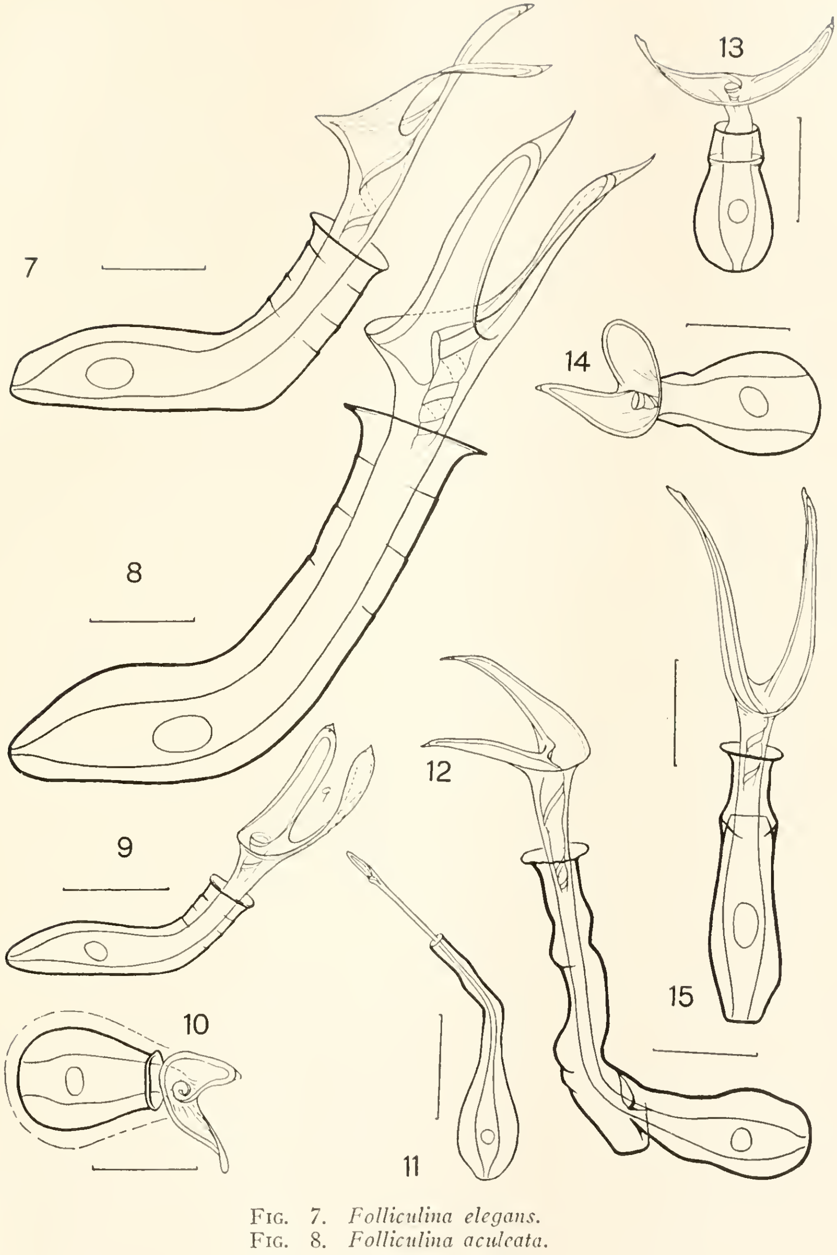

Fig. 7. *Folliculina elegans.*
Fig. 8. *Folliculina aculeata.*
Fig. 9. *Folliculina viridis.*
Fig. 10. *Folliculina simplex.*
Fig. 11. *Folliculina spirorbis.*
Fig. 12. *Parafolliculina mirabilis.*
Fig. 13. *Parafolliculina hirundo.*
Fig. 14. *Parafolliculina amphora.*
Fig. 15. *Parafolliculina violacea.*
Scale indicated = 100 μ.

Folliculina aculeata Clap. and Lach. (Fig. 8)

This species, very close to the above, is distinguished by its considerably greater size, by the development of its pharyngeal cavity and by the two acute points, protoplasmic, rigid, and very accentuated which terminate the peristomal lobes, answering completely to the description given by Claparède and Lachmann.

The infusorian measures, expanded, approximately 800 μ; the tube exceeds 550 μ; horizontal at first, it progressively bends upwards and is widely everted into a collar.

F. aculeata can be found at Concarneau at a depth of 20 meters on shells and on Bryozoa. It is probably this same species which has been described by Giard under the name of *F. abyssorum; F. similis* Dons and *F. gigantea* Dons may be synonyms, as well as *F. stylifera* Wright.

Folliculina viridis Wright (Fig. 9)

One can possibly identify with the species briefly described by Wright a small *Folliculina* whose form is very close to the two species just described, but which is very distinctly distinguished and in a constant way, by its smaller size and by its yellow-greenish or green color, very different from the blue-greenish or gray-bluish colors of all the other species.

The length of the infusorian reaches almost 400 μ, that of the lorica, 250 μ.

Folliculina simplex Dons (Fig. 10)

This tiny *Folliculina* is one of the species which have been at first associated under the name of *F. ampulla* O. F. Müller[3]; the denomination of *simplex,* which is Dons' creation, stretches out, it seems, over a group of species, distinct but very difficult to identify, because of lack of characteristic details. Various forms observed both at Concarneau and at Woods Hole seem to differ, in fact, in quite a constant manner, it may be in the shape of the constantly very short collar, or in the abundance of the blue-greenish pigment and its repartition. The only *Folliculina* known in fresh water, *F. boltoni* Kent (*Ascobius lentus* Henneguy) differs from *F. simplex* by its lodging only.

The expanded infusorian measures from 190 to 220 μ; the peristomal lobes are broad and short. The shell is patulous and measures 135–175 μ. *F. simplex* is easy to cultivate on submerged glass plates.

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[3] I first admitted this denomination, in 1932, following the description of Clap. and Lach.

Folliculina spirorbis Dons (Fig. 11)

This very characteristic species, widely distributed, which attaches itself exclusively to the tubes of *Spirorbis*, is very difficult to observe, and therefore has been described by its shell only. The shape of this shell is a kind of ampulla of about 100 μ in length, prolonged by a long and very narrow tube (11 to 12 μ), which is erect and often flexuous. The infusorian is characterized by its simplified structure; fully expanded it shows an oval body going over into a slender and very long neck, terminated by a small peristome of which the left lobe only is developed.

Genus *Parafolliculina* Dons

Macronucleus ovoidal. Shell showing an annular wrinkle, as a separation between the single plain posterior lodge and the fore-chamber, placed at the base of the neck.

Parafolliculina mirabilis Dons (Fig. 12)

In *P. mirabilis* the tube is distinctly and constantly divided in three parts: a posterior ampulla, a widened entrance and a tubular and irregular neck, sometimes bent upwards, largely everted at its margin. The total length is over 400 μ.

The expanded infusorian measures almost 600 μ; in its general shape it looks very much like *Folliculina elegans,* but the neck, which is elongate between body and peristome, is very long and slender.

This large species, very characteristic, is found in Concarneau at a depth of 15 to 20 meters in colonies of *Membranipora* and in the company of *Folliculinopsis annulata.* It may be cultivated in aquaria on immersed glass plates.

Parafolliculina hirundo Kent (Fig. 13)

P. hirundo is very similar to *Folliculina simplex,* but its horizontal shell, with a length of about 130 μ, is a trifle straighter, with an annular wrinkle limiting an anterior entrance which is but one with the very short neck. The peristomal lobes are narrow and lie relatively far apart. This small species, feebly colored, has been observed occasionally on immersed glass plates.

Parafolliculina amphora Dons (Fig. 14)

This species is distinguished from the above by the shell's aspect only, the posterior end being broader and the peristomal lobes elongated. I occasionally observed it, as well as *P. hirundo*, on immersed glass plates in aquaria, at Concarneau and at Woods Hole, Mass. The observations made on these two neighbouring forms are not sufficient to enable one to decide whether or not they should be separated into two distinct species.

Parafolliculina violacea Giard (Fig. 15)

This particularly typical and widely distributed species has been described by Laakmann (1903) and by Dons (1912) under the name of *Folliculina telesto.* The tube stands straight, about 250 μ long; the widened fore-chamber is continued by a rather short neck, everted at its margin; the entrance shows a kind of inner collar at its base.

The expanded infusorian is 500 μ long; the peristomal lobes, long and slender, stand straight and parallel to the axis of the body; the color is reddish-violet.

AN EXPERIMENTAL STUDY OF POLYSPERMY

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Although the mechanism which insures that only one sperm shall fertilize an egg is most closely concerned with the fertilization reaction as a whole, it has been very little investigated. This is largely due to the fact that no visible alteration in the surface protoplasm can be associated with the change to a fertilized condition, and the failure of the mechanism, as it appears in polyspermy—the entrance of more than one sperm into an unfertilized egg—is not easily detectable in the living condition until the time of the first cleavage. With the exception of the pioneer work on polyspermy, performed on the eggs of *Strongylocentrotus lividus* by O. and R. Hertwig in 1887, there has been little or no direct study of the phenomenon either from an observational or an experimental point of view. Numerous embryologists have been incidentally concerned with polyspermy as it appeared in the course of their work, and most of the information which has been added to the Hertwigs' contribution has come through such indirect channels. The summarized results of some of these studies are included later with the discussion of the effective agents for inducing polyspermy.

Speculation as to the nature of the mechanism which prevents polyspermy has not been wanting, but only one proposition has been supported by a majority of the authorities in the field. This explanation, embodying the conclusions reached by O. and R. Hertwig (1887), E. B. Wilson (1903), Delage (1901), Godlewski (1926), F. R. Lillie (1919) and Just (1929), while it is actually little more than a restatement of fact, includes the major points which may be deduced from the accumulated evidence. Briefly, it states that a change in the egg protoplasm, probably in the cortical region, initiated by the fertilizing sperm, renders the egg inaccessible to further sperm. It is generally agreed, moreover, that polyspermy is caused by a retardation in the rate at which this change is propagated around the egg from the entrance point of the sperm.

Until the present, this latter proposition has stood unchallenged by experimental procedure, although it is actually only a deduction from scattered qualitative observations. While it is admittedly the simplest and most easily visualized mechanism which can be postulated to explain the observed effects, such reasoning is insufficient foundation for the confidence and lack of critical examination which it has enjoyed. Even such preliminary experimentation as is reported in this paper leads to results which are exceedingly difficult to reconcile with the idea of the retardation of a propagated change in the egg cortex as the cause of polyspermy. It is entirely possible that the true explanation may have an entirely different basis, and this possibility should at least be kept under consideration.

In view of the fact that monospermy is one of the few general characteristics of fertilization which are capable of manipulation without immediately fatal consequences, it is rather remarkable that so little attention has been given to its study as a method of investigating the fertilization reaction. At the suggestion and under the direction of Dr. L. V. Heilbrunn, the following experiments were undertaken to repeat with the eggs of *Arbacia punctulata* the work done by the Hertwigs (1887) on *Strongylocentrotus*. Their original list of agents was extended to include a wider range of chemical substances, and a few studies were made on other species for comparison.

General Method

In general, one procedure was used in all the succeeding experiments, with slight modifications in certain cases. The eggs and sperm were obtained from the animals according to the directions given by E. E. Just (1929). The oral surface of the sea urchin was cut, the lantern removed, and the male animals inverted over dry Syracuse watch glasses, while females were allowed to shed into Stender dishes containing sea water. After being washed several times in sea water, some of the eggs from each female were tested by insemination. Such lots as gave 100 per cent entirely normal fertilization reaction were used for experimentation. In some cases several lots were mixed (usually eggs from 2–5 females) in order to reduce the variation in the results arising from individual differences characteristic of the parent animals.

The number of sperm used for insemination in each experiment was controlled so far as possible by making a standard suspension consisting of one drop of "dry sperm" in 25 cc. of sea water. From 2–5 drops of this suspension were used to fertilize each lot of eggs in the experiments. Although this method gives only an approximately uniform insemination, experiments showed that even a much wider range of variation had in itself no effect in changing the number of sperm which entered each egg. Except in experiments involving over-insemination, only enough sperm suspension was used to produce a moderate insemination—approximately 1/20 of the sperm concentration which was necessary to secure 10 per cent polyspermy in otherwise untreated *Arbacia* eggs.

In the case of each reagent, tests were made with dilute sperm suspensions to make sure that the sperm retained their complete capacity for fertilization after exposures of at least 5 minutes. It was assumed that within this time all fertilizable eggs in a well-mixed sperm and egg suspension would be fertilized.

The general procedure in all experiments was as follows: eggs and sperm were shed, and the eggs tested for 100 per cent normality. Several drops of concentrated egg suspension were introduced into the test solutions and left for varying periods. The eggs were inseminated in the test solution, or they were removed to sea water containing sperm, or to sea water to which sperm were added after a short recovery period. Controls were inseminated in sea water to check the incidence of polyspermy resulting from injury incurred during the handling of the eggs. In almost no case was this higher than 1–2 per cent, and a majority of the cases showed none at all.

The eggs were allowed to develop to the first cleavage, and the percentages of development (i.e., partial or complete fertilization—raised fertilization membrane, characteristic change in pigmentation, or visible nuclear activity) and of polyspermy (cleavage into three or more blastomeres or appearance of three or more daughter nuclei in an uncleaved egg after an hour) were recorded. The percentage of development represents the ratio of activated eggs to all the eggs in the suspension. The figures for polyspermy, on the other hand, give the percentages of *activated eggs* which were polyspermic.

In certain cases in which the test solution was strongly toxic it was removed after 10–15 minutes and fresh sea water was added, to allow the development of the eggs to proceed. This was done only when sufficient time had elapsed after insemination so that all the sperm in the suspension had been deprived of fertilizing power.

In these experiments, although the temperature varied during the season from 18.0° to 28.0° C., most of the work was done at about 23.0° C. In any one experiment all the solutions were allowed to come to the same (room) temperature before the eggs were introduced into them. Undoubtedly some of the variation observed in the results may be traced to temperature effects; in order to separate out such variation from that due to individual differences, it will be necessary to perform a series of experiments using eggs from the same females at extremes of the temperature range. In the meantime no consistent effect can be detected in the data at hand.

All alkaloid solutions were made up in fresh sea water, checked for pH with Clark and Lubs indicators, and kept in a refrigerator between experiments to prevent decomposition of organic constituents. Other conditions, such as amount of solutions and number of eggs, were kept relatively constant by the use of Stender dishes and pipettes of uniform size, and results were checked with controls whenever this was possible.

SPECIFIC DIFFERENCES IN SUSCEPTIBILITY TO POLYSPERMY

It is generally conceded throughout the literature that insemination with an excess of sperm will cause polyspermic fertilization in the eggs of many marine animals, even when these eggs are in good condition. Just (1929), on the other hand, directly contradicts this. In deciding the issue, there is always the difficulty of determining the point at which the concentration of sperm begins to inflict injury upon the egg cells by accumulation of CO_2 and probably other substances. In *Arbacia* such injury is very apparent with extremely heavy insemination, causing marked irregularities in development. The polyspermy which appears with moderately heavy sperm concentrations may well be the first evidence of the injury. On the other hand, great differences in the susceptibility of eggs to polyspermic fertilization can be demonstrated between different species. *Cumingia* eggs, for example, are strongly affected by sperm concentrations which would give no polyspermy in *Arbacia*, and which, on microscopic examination, show no crowding of sperm about the egg peripheries.

A technique for exact comparative study of different species would require the development of a special method for counting with some accuracy the number of sperm in a given suspension. There are, moreover, other factors, such as size of eggs, size and shape of sperm, and the precise mechanism for sperm entrance into the eggs, which contribute to the final result. With these qualifications in mind, a simple method was used to give approximate values for each of the four species studied. For *Arbacia punctulata* and *Asterias forbesii*, sperm suspensions could be made roughly comparable by using the same dilutions of dry sperm. Preliminary experiments indicated the sperm concentration which uniformly gave 100 per cent fertilization with the lowest percentage of polyspermy to be 0.06 cc. of the "standard suspension" made by mixing one drop of dry sperm with 25 cc. of sea water. With lighter insemination not all the eggs were fertilized; when the amount of sperm suspension was considerably increased (ca. 10 times), polyspermy became slightly more frequent. The sperm of *Cumingia tellinoides* and *Cerebratulus lacteus* cannot be obtained dry; the procedure used in these cases was to determine the lowest concentration which consistently produced 100 per cent development when the eggs were inseminated with 0.06 cc. of it. This then formed the unit suspension of which increased amounts were used to produce polyspermy.

Eggs and sperm of *Arbacia* were obtained in the usual manner. *Asterias* eggs were procured by removing one-half of a ripe ovary to a finger-bowl filled with sea water. After 5 minutes the ovary was removed; the eggs which had been extruded were allowed to remain in the sea water for from 30 to 55 minutes to permit the breakdown of the germinal vesicle. Sperm were shed into a dry Syracuse watch glass from the excised half-testis, and the test suspensions made from this concentrated stock. *Cumingia* eggs and sperm were shed into Stender dishes of sea water into which the animals were placed after being kept dry for a short time. *Cerebratulus* gametes were teased out of sections cut from the sexually mature worms. In order to permit a rough comparison of the results, the sperm concentration obtained by adding 0.06 cc. of the "standard suspension" to an approximately equal number of eggs in 30 cc. of sea water is considered as unity in Fig. 1. The eggs were inseminated with multiples of this amount to 100 times the unit suspension or until the incidence of polyspermy approached 100 per cent.

The curves for the four species are plotted in Fig. 1. It is at once apparent that there are wide differences in the order of susceptibility to polyspermy among these species. When the unit concentration of *Cerebratulus* sperm is increased 20 times, these eggs are nearly all polyspermic. Because of the difficulty of obtaining eggs and sperm of this animal, the experiment was performed only once, and the result must be considered as simply indicative. *Cumingia* eggs are 100 per cent polyspermic with 50 times the original sperm concentration, while a sperm suspension 75 times more concentrated than the minimum gave only 41 per cent polyspermy in *Asterias* and 14 per cent in *Arbacia* eggs. No correlation can be found between the size of the egg and susceptibility to polyspermy, the size series being *Cerebratulus*, *Asterias*, *Arbacia*, *Cumingia*. It is most probable that the explanation of this disparity must be sought, not in morphological or simple physical and chemical variations, but in the less easily defined physiological differences between various types of eggs, such as their resistance to deterioration after exposure to sea water, and the influence which is exerted on the cytoplasm by the stage of the maturation process in which the eggs are fertilized.

AGE

Preliminary studies of the effect of aging on *Arbacia* and *Asterias* eggs were undertaken to furnish data for comparison between the two forms, and also to attempt to find a basis for explanation of the fact that Smith and Clowes (1924), in their pH work, obtained 100 per cent polyspermy in *Asterias* at the pH of sea water. In these experiments no attempt was made to control temperature or to check bacterial action, since the aim was not to differentiate between the rôles of various factors in the aging process, but to discover their combined effect on the fertilization reaction.

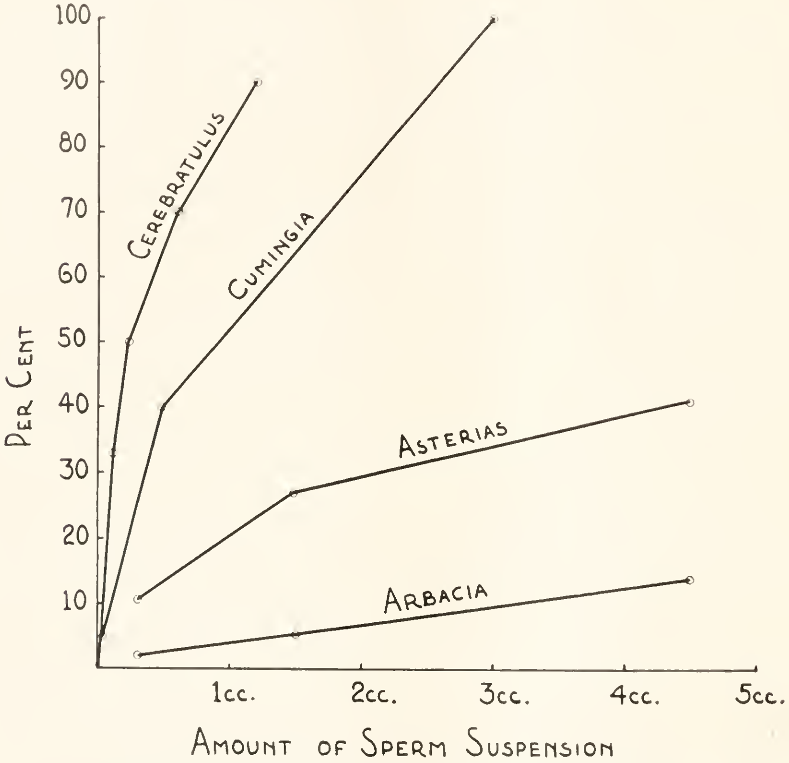

FIG. 1. Relative susceptibility of four species to polyspermy caused by over-insemination.

Eggs were kept in uncovered finger-bowls filled with sea water, in dilute suspension to avoid undue crowding, at room temperature. They were removed at intervals to Stender dishes and inseminated with freshly secured sperm in the usual concentration.

Arbacia eggs uniformly showed no diminution in their resistance to polyspermy with moderate insemination until they had been standing at least 16 hours in sea water. The percentage of polyspermy then increased regularly until at 23 hours it occurred in all eggs which were still capable of fertilization. The eggs of *Asterias* presented a very different picture, which is represented in graphic form in Fig. 2. These curves, which indicate the increased polyspermy with increased time after shedding of the eggs into sea water, include the results of 5 experiments in which eggs were inseminated with the usual light sperm suspension, and simultaneously with one 80 times more concentrated. When eggs are inseminated lightly 45 minutes after exposure to sea water, the polyspermy is somewhat less than 10 per cent; after exposures of 85 minutes to sea water, the polyspermy increases to 24 per cent. Between 2 and 2½ hours after shedding, 86 per cent of the eggs are polyspermic, even though very low concentrations of sperm are used. With heavy insemination, there is 36 per cent polyspermy at about 45 minutes, 73 per cent after 85 minutes, and 100 per cent between 2 and 2½ hours. Microscopic examination of heavily inseminated eggs immediately after insemina-

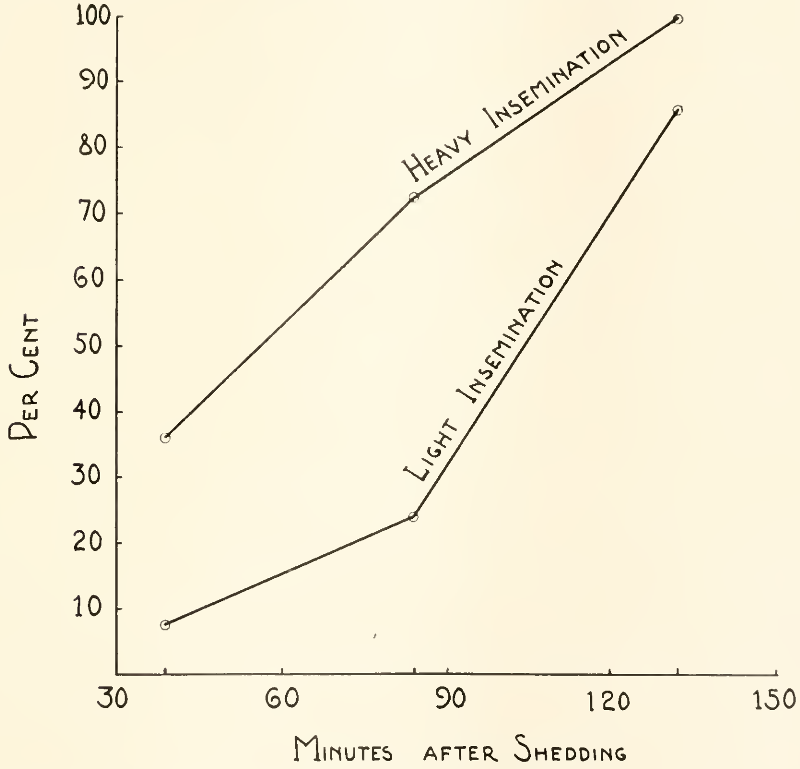

Fig. 2. Increasing susceptibility to polyspermy with increased time of standing in sea water, in the eggs of *Asterias forbesii*.

tion showed the jelly surrounding the eggs to be completely filled with active sperm. This degree of insemination must be comparable to that designated by Smith and Clowes as "maximum" since it approaches the physiological limit—further increase in the number of sperm causes injury apart from polyspermy.

The rapidity with which *Asterias* eggs lose their capacity to resist polyspermy after exposure to sea water and completion of the maturation process suggests that the high percentages of polyspermy found by Smith and Clowes are due to the use of eggs which had stood in sea water too long (i.e., $1\frac{1}{2}$–2 hours) after shedding to be in optimum condition for fertilization.

HERTWIG REAGENTS

In repeating with *Arbacia punctulata* the Hertwig (1887) experiments performed on the eggs of *Strongylocentrotus lividus* (*Paracentrotus lividus*), no attempt was made to determine the optimum concentration or length of exposure for the production of polyspermy with the particular substance used, chiefly because the present state of the knowledge both of the mode of action of the complex chemicals involved and of the cell mechanism affected by them is too fragmentary to make profitable use of detailed quantitative data. In each case the concentrations selected in the original work were used, although the time of exposure was varied to some extent. In order to overcome the fluctuations introduced by individual variation, the data presented include results of several experiments performed on different lots of eggs. Except in cases in which the percentages were close to 0 or 100, the final average is made from individual experiments, the results of which may vary widely. Indeed, the average for one lot of eggs may be ± 30 per cent from the final average.

The substances which were selected, somewhat fortuitously, by the Hertwigs for their experimental studies of fertilization fall rather clearly into three categories so far as their action on *Arbacia* egg cells is concerned. Chloroform, which is in a class alone, will be discussed below. Of the others, chloral hydrate, cocaine hydrochloride and nicotine form one group, and morphine sulphate, strychnine sulphate and quinine sulphate, another.

The action of the first group is characterized in general by its lack of effect in entirely blocking fertilization, its production of a high percentage of polyspermy, and its immediate and nearly complete reversibility on return of the eggs to sea water for insemination. The substances in the second group tend to reduce the percentage of development; they induce less polyspermy, and the polyspermy-inducing action is not so rapidly reversed on return to sea water, although the block to fertilization is removed. (See Table I.) Chloral hydrate, cocaine hydrochloride and nicotine, in concentrations which will give 100 per cent polyspermy, permit development to proceed at least past the first few cleavages in the test solutions, while morphine, strychnine and quinine sulphates are rapidly (within 30 minutes after insemination) fatal to eggs left to develop in the solutions, turning the protoplasm dark and apparently coagulating it. These facts seem to indicate a more or less specific attack, on the part of the substances in the first group, on the mechanism of monospermy, while the second group of substances injures the whole cell, and polyspermy is only one of the manifestations of its poisoned condition.

TABLE I

Comparative resumé of results obtained by treating unfertilized *Arbacia* eggs with reagents used by O. and R. Hertwig (1887).

Reagent	Inseminated in solution		Inseminated in sea water	
	Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Chloral hydrate, 0.2%	98	92	100	5
Cocaine HCl, 0.025%	95	80	100	19
Nicotine, 1 drop—200 cc	94	73	100	9
Morphine SO_4, 0.6%	53	52	100	31
Strychnine SO_4, 0.1%	100	56	100	50
Quinine SO_4, 0.05%	76	31	100	76

Although these common characteristics are apparent, the action of each of the reagents is sufficiently different from the others to warrant a brief individual description.

Chloral Hydrate

This substance, in 0.2 per cent solution, gives a higher percentage of polyspermy than any of the alkaloids, with apparently less injury to the cells, after 5 minutes exposure or less (see Table II). Its action is initiated very rapidly, as is shown by the fact that eggs introduced into a chloral hydrate solution containing sperm were 88 per cent polyspermic. It is very interesting that almost the maximum effect is obtained during the exceedingly brief interval which intervenes between contact of egg cell and solution and arrival of sperm at the egg surface. It is possible, in these experiments, that the sperm-excluding mechanism was secondarily broken down as a result of exposure to the solution, permitting the entrance of more sperm into

TABLE II

Results obtained from treatment with 0.2 per cent chloral hydrate.

Exposure	Number of animals	Inseminated in solution		Inseminated in sea water	
		Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
minutes					
0	7	99	88		
1	7	98	97	100	3
5	7	96	97	100	7

normally fertilized eggs—however, there seems to be at present no method of checking this point. In preliminary experiments with nicotine, attempts to secure the entrance of extra sperm into normally fertilized eggs by exposing them to solutions of the drug a few minutes after fertilization gave negative results, even when the fertilization membranes were shaken off the eggs.

With a greater concentration of chloral hydrate—0.5 per cent—the effect on the cell is less specifically the production of polyspermy and more a general toxicity—i.e., the percentages of both development and polyspermy are reduced, and the immediate reversibility in fresh sea water is lost (see Table III).

TABLE III

Results obtained on treatment with 0.5 per cent chloral hydrate.

Exposure	Number of animals	Inseminated in solution		Inseminated in sea water	
		Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
minutes					
0	6	35	61		
1	6	73	97	100	6
5	6	90	100	100	45
10	6			100	70

Cocaine Hydrochloride

Cocaine HCl in 0.025 per cent solution is similar to 0.2 per cent chloral hydrate in its action, giving high percentages of development and polyspermy. Its effect is found to be almost completely reversed when treated eggs are inseminated immediately after their return to sea water (see Table IV).

TABLE IV

Treatment with 0.025 per cent cocaine hydrochloride.

Exposure	Number of animals	Inseminated in solution		Inseminated in sea water	
		Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
minutes					
0	18	90	51		
2	18	100	73	100	10
5	18	99	73	100	4
10	18	100	82	100	11

Nicotine

The nicotine solution was made by shaking one drop of nicotine with 200 cc. of sea water. In this concentration nicotine is almost identical with 0.025 per cent cocaine hydrochloride in effectiveness and reversibility (see Table V).

TABLE V

Results obtained on treatment with nicotine—1 drop of extract in 200 cc. sea water.

Exposure	Number of animals	Inseminated in solution		Inseminated in sea water	
		Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
minutes					
0	7	80	56		
1	12	100	66	100	3
5	7	99	97	100	5
10	9	93	87	100	15

Qualitative experiments show that nicotine is effective over a very wide range of concentrations, from 1 drop in a liter of sea water to 50 drops in a liter. The close correlation between severity of treatment and the resulting degree of polyspermy is beautifully shown over this concentration range, although no quantitative measure can be given because of the impossibility of establishing exact grades of polyspermy. However, the progression is very apparent, from scattered cases of two-sperm fertilizations with the lowest concentrations of nicotine, through a point at which there is a general occurrence of di- and trispermy. Then, with stronger concentrations, fertilization uniformly involves 4 or 5 sperm, and finally, with very strong concentrations, there is entrance of many (ca. 20 or more) sperm into all eggs, with

TABLE VI

Eggs exposed to 0.6 per cent morphine sulphate for 15 minutes.

Inseminated in	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Solution	7	53	52
Sea water immediately after transfer	7	100	31
Sea water after 1 minute	7	100	1

subsequent cleavage into many blastomeres after the first hour, or entire suppression of cleavage, with the cells filled with sperm nuclei.

Low concentrations of nicotine solution (e.g., 3 drops in a liter of sea water) also permit clear demonstration of the reciprocal rôles of time and concentration. When eggs are exposed to a given solution for increasing periods, the same gradation of effect is obtained, although the extent of the range is usually restricted, i.e., with low concentrations even long exposure will not give the maximum effect, and the shortest possible exposure to a high concentration will produce more than the minimum effect.

Morphine Sulphate

In the second group of substances, morphine sulphate is most like the members of the preceding group in that there is some immediate recovery from its effects upon return of the eggs to sea water, and complete recovery takes place within one minute (Table VI). The percentage of development is, however, reduced to one-half when the eggs are inseminated in the solution, and polyspermic fertilization occurs at best in only about fifty per cent of these fertilized eggs. In order to obtain development as far as the first cleavage, it was necessary to remove the test solution and substitute sea water.

Strychnine Sulphate

The action of strychnine is less generally toxic than that of morphine, permitting 100 per cent fertilization (see Table VII), but its

TABLE VII

Eggs exposed to 0.1 per cent strychnine sulphate for 5 minutes.

Inseminated in	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Solution	13	100	56
Sea water immediately after transfer	13	100	50
Sea water after 1 minute	13	100	17

capacity for the production of polyspermy is less rapidly reversible in fresh sea water, showing almost no diminution on insemination in sea water immediately after transfer from the strychnine solution. There is only partial recovery of normal fertilizability after 1 minute.

Quinine Sulphate

The tendency toward irreversibility is carried to a remarkable extreme in the action of quinine sulphate, which shows a more than 100 per cent increase in the incidence of polyspermy when the eggs are transferred to sea water containing sperm, after 10 minutes or less exposure to a 0.05 per cent quinine sulphate solution (see Table VIII). Even after 1 minute of recovery in sea water, the percentage of polyspermy is higher than that obtained by insemination in the solution. This result is wholly unexpected and at present inexplicable.

TABLE VIII

Eggs immersed in 0.05 per cent quinine sulphate solution for 10 minutes.

Inseminated in	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Solution	12	76	31
Sea water immediately after transfer	16	100	76
Sea water after 1 minute	9	100	42

It is impossible to draw comparisons between these results and those of the Hertwigs because differences between the backgrounds of knowledge, the points of view and the methods employed in the two studies place the data in different categories. The most that can be said is that the agents studied affect the eggs of *Paracentrotus lividus* and of *Arbacia punctulata* in the same direction, and to an approximately similar extent.

FAT SOLVENTS

During the course of two summers, experiments were performed to test the action of fat solvents—ethyl alcohol, ether, chloroform and ethyl urethane—in causing polyspermy. It was expected that these substances would have well-marked effects because of their anæsthetic properties. Solutions were made up in every case immediately before their use, and kept covered while the eggs were being exposed to their action before insemination. The optimum concentrations for the production of polyspermy were: ether, 2.0 per cent; ethyl alcohol, 3.0–5.0 per cent; chloroform, 0.2 per cent; ethyl urethane, 2.0 per cent.

The results of these experiments were so varied that many more data and more exact control of experimental conditions will be necessary before significant values can be obtained. From the results at hand, however, several facts are apparent. In the first place, all the substances tested are effective agents for the production of polyspermy, and, so far as could be determined, they possess roughly the same order of effectiveness. Experiments which involved insemination in sea water after exposure to the solution showed that the action of all the substances is reversed immediately upon return to sea water unless the treatment has been prolonged or severe.

Probably the greatest part of the observed variation can be ascribed to the temperature effect which is easily detectable in the data, although no experiments were planned to test this point. The fact that one concentration of ether or ethyl alcohol, over the temperature range from 22.0° to 27.0° C., caused successively no observable effect, 100 per cent polyspermy and 100 per cent cytolysis of the cell protoplasm demonstrates clearly that the treatment resulting specifically in the production of polyspermy possesses a very narrow range of effectiveness which lies only slightly below the lethal dose.

Common Cations

The extensive work which has been done by R. S. Lillie (1910), J. Loeb (1915), and others on the effects of the sodium, potassium, magnesium and calcium ions on egg cells and on the fertilization reaction suggested an attempt to discover the possible rôle of these cations in the production or prevention of polyspermy. Because they are constituents of the medium in which fertilization normally takes place, and because their properties are relatively well defined and their action on single cells has been extensively studied, they are more useful agents for experimentation on egg cells than the more complex and less familiar alkaloids. Several factors are involved, however, and these complicate the situation.

The greatest difficulty lies in the fact that the eggs cannot be fertilized in pure isotonic solutions of the chlorides of these ions because such solutions are highly toxic to both eggs and sperm. Moreover, it is impossible, without using a centrifuge to concentrate the eggs, to carry them from sea water to a pure chloride solution without including traces of the other salts of sea water. Centrifugal force was not used because of the danger of mechanical injury to the eggs.

With the possibility of inseminating in pure solutions ruled out, two alternative methods were used: first, exposure of eggs to chloride solutions for a relatively long time (20 minutes) and insemination immediately upon return to sea water; and second, the addition of an excess of the isotonic salt solution to sea water and insemination in this mixture. Since the most drastic effect of isotonic solutions of sodium, potassium and magnesium on sperm motility depends on the lack of calcium, and since calcium is effective in extremely small quantities, the sperm retain their motility and fertilizing power in such solutions containing a great excess of one ion, which must then be considered to be exerting the predominant effect in influencing the course of the fertilization reaction.

Experiments with Isotonic Chloride Solutions

Arbacia eggs were shed into sea water and washed in the usual fashion; they were later transferred through three dishes of isotonic salt solution within twenty minutes, and inseminated immediately upon return to sea water. Although the shedding of the eggs directly into the salt solutions would have eliminated the necessity for repeated washings and insured entire absence of the other constituent salts of sea water, this procedure was not adopted because previous experimentation had shown that the percentage of development was much reduced in eggs which were not washed in sea water immediately after shedding and before other treatment was begun. In these experiments the eggs were concentrated as much as possible before transference from one solution to the next; however, it is certain that traces of all the common constituents of sea water were carried through the washings. The use of the hydroxides of sodium and potassium to raise the pH of calcium and magnesium solutions to that of sea water also introduced a slight error. The solutions, then, cannot be considered as quantitatively pure, but the marked differences found to exist between the actions of such solutions of the four common cations indicate that characteristic effects are being produced. Isotonic solutions were made with Merck's c.p. chemicals, NaCl and KCl in 0.53M solution and $CaCl_2$ and $MgCl_2$ in 0.32M solution. (The latter solutions were slightly hypertonic.) The pH was adjusted with 0.1N NaOH and KOH.

TABLE IX

Unfertilized eggs washed for 20 minutes in three changes of isotonic solution, inseminated in sea water.

Cation	Number of experiments	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Na	13	34	100	48
K	21	64	96	35
Ca	9	19	97	19
Mg	14	42	100	13

The results of these experiments are summarized in Table IX. It must be emphasized again that these figures are not indicative of the absolute magnitude of the polyspermy-producing action exerted by these reagents, since fertilization did not take place in the test solutions. They probably represent the amount of such effect which was not immediately reversed upon return to sea water, since, with the single exception of quinine sulphate, all the reagents tested gave a higher percentage of polyspermy when insemination took place in the solution than when the treated eggs were changed to fresh sea water containing sperm.

Experiments with Salt-Sea Water Mixtures

Mixtures of sea water and isotonic salt solutions were made in varying proportions, and eggs inseminated in these test solutions after an exposure of 10 minutes. A condensed comparison of the results obtained by this procedure with sodium, potassium, magnesium and calcium chlorides is presented in Table X. It is strikingly apparent, from the distribution of the italicized values in the table indicating the approximate points at which the percentages of development were reduced by one-half, that the four cations have very unequally toxic effects on the fertilization reaction. This toxicity is not, however, clearly correlated either directly or indirectly with the capacity of the salts to induce polyspermy. It is perhaps significant that the least toxic, magnesium, produces the most polyspermy; on the other hand, sodium, which is only slightly more injurious than magnesium, is entirely ineffective so far as polyspermy is concerned. It is interesting, also, to note that this procedure gives results which are almost the opposite of those obtained by exposing eggs to solutions of single chlorides.

TABLE X.

Effect of 10-minute exposure to salt-sea water mixtures. Percentages represent salt solution/sea water ratio. *D* = percentage of all eggs activated; *P* = percentage of all activated eggs polyspermic.

Salt	No.	17%		33%		50%		67%		80%		93%	
		D	P	D	P	D	P	D	P	D	P	D	P
NaCl	12			100	2	100	0	94	0	*58*	0	10	3
KCl	12	*50*	0	31	0	0	—						
$CaCl_2$	7	94	5	62	1	*40*	2	8	6	0	—		
$MgCl_2$	60	100	5	98	9	93	17	98	37	87	71	*44*	32

The data accumulated for magnesium in the course of a large number of experiments show that the concentration of magnesium (4 parts isotonic $MgCl_2$–1 part sea water) which causes the highest percentage of polyspermy is precisely the amount at which the fertilizability of the eggs begins to drop off sharply (see Fig. 3). This relationship points to the conclusion that the magnesium-sea water solution affects the sperm-excluding mechanism just before it fatally injures the cell as a whole.

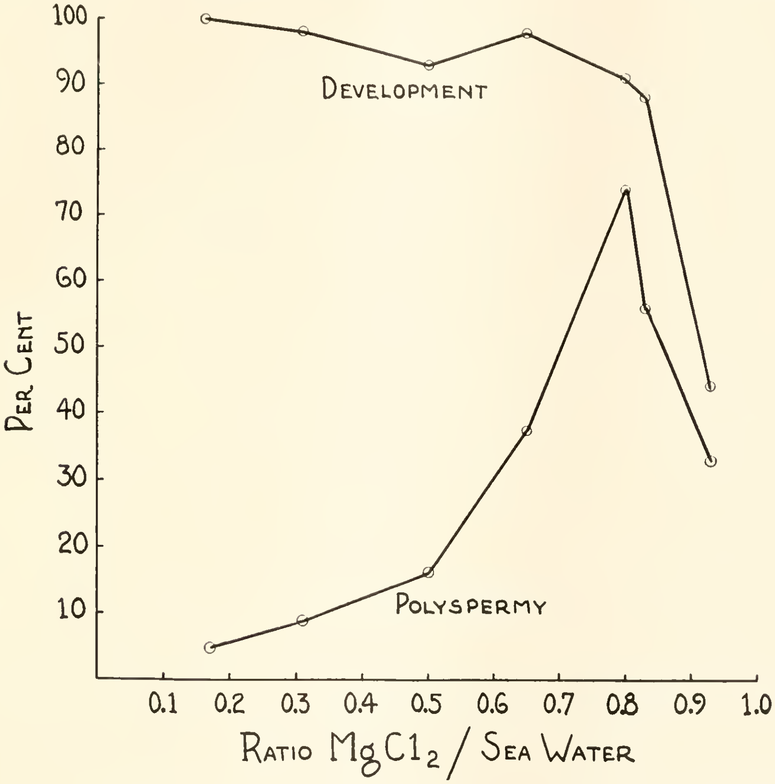

FIG. 3. Effect of various proportions of isotonic $MgCl_2$ and sea water on development and polyspermy in *Arbacia punctulata.*

In an attempt to discover why the proportion of one part sea water to four parts $MgCl_2$ was more effective than any other combination, a series of experiments was performed in which one part of isotonic NaCl, KCl and $CaCl_2$ respectively was added to four parts of $MgCl_2$. The results are shown in Table XI. The fact that the mixture of $MgCl_2$ and NaCl has an effect so similar to that of $MgCl_2$ and sea water is not surprising in view of the predominant amount of sodium in sea

Table XI

Unfertilized eggs exposed for 10 minutes to isotonic salt mixtures.

24 cc. $MgCl_2$ + 6 cc. of	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
NaCl	20	91	71
KCl	20	25	11
$CaCl_2$	20	22	50
Sea water	60	91	72

water. With the small traces of calcium introduced into the solution as part of the sea water in which the eggs were suspended, the final solution contains the cations necessary for fertilization. From the work of Fukuda (1935) it appears that characteristic changes in the permeability of sea urchin eggs to water follow exposure to mixtures of sodium and magnesium. It is highly probable that other functions of the cell protoplasm besides its permeability to water are affected by such ionic interaction, and it is entirely possible that the conditions leading to polyspermy may depend on similar combination effects. This seems the more probable when the ineffectiveness of magnesium chloride alone is considered.

pH

Because Smith and Clowes (1924) have found changes in the pH of sea water to be an exceedingly effective method of causing polyspermic fertilization, a series of experiments was performed on the eggs of *Arbacia punctulata* to repeat this work with the same procedure as that used in the preceding studies, in order to obtain data which

Table XII

Effect of 10-minute exposure to sea water at lowered pH. Eggs inseminated in pH solutions.

pH	Number of experiments	Percentage of all eggs activated	Percentage of activated eggs polyspermic
5.8	5	4	20
6.4	9	49	46
7.0	24	91	78
7.6	9	97	50
8.2	9	99	7
Sea water	24	99	0–2

would be strictly comparable with the values obtained for the other agents. This work differed from Smith and Clowes' in that counts were made at the time of the first cleavage instead of 2–4 hours after insemination, and treated eggs were inseminated with a light suspension of sperm (see above) instead of the "excess sperm" used by Smith and Clowes. In other respects their technique was followed exactly. The sea water buffers were broken down by the addition of 23 cc. 0.1N HCl/liter of sea water, and the water was allowed to stand overnight in a shallow dish to permit escape of the excess CO_2. The pH was adjusted by the addition of 0.1N NaOH, and checked with Clark and Lubs indicators. Eggs were inseminated in the solutions after 10 minutes exposure.

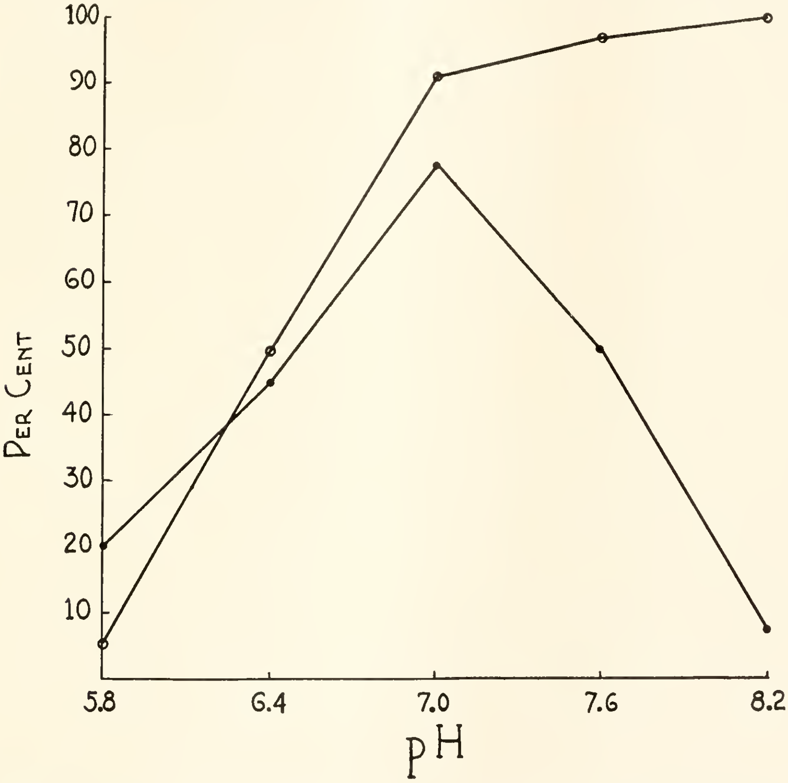

FIG. 4. Effect of pH on development and polyspermy in *Arbacia punctulata*. Open circles, development; solid circles, polyspermy.

The results, as shown in Table XII, correspond well with those of Smith and Clowes for *Arbacia*, when allowance is made for the lighter insemination. It is interesting to note that the percentage of polyspermy is perceptibly increased over the normal sea water control when the carbonate buffer system is broken down and the pH brought back to 8.2 by the addition of NaOH. As reported by Smith and Clowes, the greatest incidence of polyspermy occurs at about neutrality. As they are plotted in Fig. 4, the data show a somewhat wider range over which polyspermy occurs, as well as a more gradual rise both to 100 per cent fertilization and to the maximum amount of polyspermy.

DISCUSSION

As in the case of activation of egg cells, the range of effective agents for the production of polyspermic fertilization in sea urchin eggs is exceedingly wide and bewilderingly contradictory. Simple explanations are immediately ruled out by data proving the efficacy of an agent which increases the rate of metabolism—heat, and one which decreases it—cold; of an excitant—strychnine, and a depressant—alcohol; of a substance which raises permeability—Na, and a substance which lowers it—Mg.

A list of the agents or conditions which have been shown to produce polyspermy is given here, to indicate the wide field which they cover.

AGENT	INVESTIGATOR
Physico-chemical	
Heat	Hertwig, O. and R., 1887
Cold	Hertwig, O., 1890 Bury, J., 1913
Acid sea water (pH 7.0–7.2)	Smith and Clowes, 1924 Rose, M., 1931
Egg and sperm extracts	Sampson, M., 1926 Godlewski, E., 1934
Common cations	This paper
Alkaloids	Hertwig, O. and R., 1887
Fat solvents	
CH_3Cl	Hertwig, O. and R., 1887
Ether, ethyl alcohol, ethyl urethane	This paper
Chloral hydrate	Hertwig, O. and R., 1887
Physiological	
Mechanical injury	Hertwig, O. and R., 1887
Age	Hertwig, O. and R., 1887
Immaturity	Fol, H., 1877
Overripeness	Fol, H., 1877
Poor condition of parent animals	Fol, H., 1877 Hertwig, O., 1890
Overinsemination	Gemmill, J. J., 1900

It is very probable that further work will show still more diverse substances to be equally effective, since the effort to extend the list in the past has been largely directed by one theory—that polyspermy is caused by substances or conditions which tend to slow up the speed of some cortical reaction. When this point of view is disregarded, other agents can be added to the list which may in time contribute to a generalized concept and to further analysis of the steps in the fertilization reaction.

One fact which is of undoubted importance in connection with an understanding of experimentally induced polyspermy is that in very many cases a direct correlation can be observed between the severity of the treatment—time of exposure × concentration of reagent—and, at first, the number of eggs which is polyspermic and later, when they are all affected, the number of sperm which enter each egg. The significance of this point lies in the fact that it definitely disproves the existence of an all-or-none type of mechanism, and indicates rather a reaction which may be retarded or accelerated by the treatment.

From the fact that the shortest possible time of exposure to most of the reagents gave at least half as much polyspermy as much longer exposures, it is evident that the reaction which is responsible for the appearance of polyspermy is initiated very rapidly. The complete reversal on return to sea water which was observed with optimum treatment (e.g., 0.2 per cent chloral hydrate) again emphasizes the rapidity of the process, besides indicating its reversibility. It is necessary to point out in this connection that the interval between introduction of the eggs into a given solution containing sperm and their fertilization consists in little more than the time which it takes for the sperm to penetrate the egg jelly, since the sperm are well dispersed and must reach the eggs as soon as they enter the solution.

The uniformly short time of exposure which is effective with all reagents in spite of the fact that the living cell membrane intervenes between the egg protoplasm and its environment, and in spite of the very different rates of penetration into cells of the various substances involved, would lead to the supposition that whatever mechanism is affected lies at or immediately beneath the cell membrane. This is, of course, the location which would be postulated for a reaction that is concerned with stimuli entering the cell from the outside, and which is deranged by treatment that leaves other cell functions apparently normal.

The reversibility after moderate, and the irreversibility after severe treatment with chemical agents is an important characteristic of experimental polyspermy, and one which will materially assist in defining the reaction by excluding many types of effects which are known to be consistently irreversible, and by affording an index of severity of treatment. The anomalous case of quinine SO_4, which doubles its effectiveness on return to sea water instead of reversing to normal, provides interesting material for speculation, but it cannot be considered to invalidate the other results since so little is known of the actual mode of action of this substance.

It is certainly apparent that the information at hand is insufficient basis for any final conclusion concerning the mechanism of monospermy. However, as working hypotheses, some of the possibilities which these data suggest can be used to advantage in a further attack on the problem. For example, it is known that Na, K, Mg (Heilbrunn and Daugherty, 1932), ether (Heilbrunn, 1934), and cold (Thornton, 1935) cause a change in the viscosity of cortical protoplasm in the direction of liquefaction. All these agents have been shown to be more or less effective agents for causing polyspermy. In view of the fact that a marked gelation of the sea urchin egg cortex occurs at fertilization (surface pigment granules which are easily moved by centrifugal force before fertilization cannot be dislodged immediately afterward), it is possible that interference with this gelation would disturb the supernumerary sperm-excluding mechanism to the extent of producing polyspermy.

Another observable change in the physico-chemical state of the sea urchin egg cortex which is closely connected in some way with the fertilization reaction is the shift in color of the egg surface with dark-field illumination, as reported by Runnström (1928). The color has been shown to be caused by the lipoid constituents of the outer membrane. Dark-field observations of treated eggs with special attention to the coloration of the lipoid layer would determine whether or not the change in the coloration of the lipoid layer seen by Runnström is important in connection with monospermy.

This study, then, has shown that the problem of polyspermy can be attacked by measures which will yield quantitative results, and it is possible that data from this source may constitute a significant addition to the knowledge of the fertilization reaction.

SUMMARY

1. Experimental study of pathological polyspermy is discussed as a method for ascertaining the dynamic characteristics of the fertilization reaction and especially of monospermy.

2. A general procedure is described for treatment of unfertilized

eggs, standardization of sperm suspensions, and determination of percentages of polyspermic fertilizations.

3. Comparable overinsemination of the eggs of *Arbacia punctulata*, *Asterias forbesii*, *Cumingia tellinoides* and *Cerebratulus lacteus* shows sharply decreasing resistance, in the listed order, to polyspermy caused by excess sperm.

4. Eggs of *Asterias forbesii* become susceptible to polyspermic fertilization after standing in sea water less than two hours. Eggs of *Arbacia* show little diminution in their normal resistance to polyspermy until after sixteen hours in sea water.

5. Unfertilized *Arbacia* eggs were exposed to dilute solutions of chloral hydrate, cocaine hydrochloride, nicotine, morphine sulphate, strychnine sulphate and quinine sulphate. The effectiveness of these substances in inducing polyspermy is discussed and compared. The results for *Arbacia punctulata* are found to be qualitatively similar to those obtained by O. and R. Hertwig (1887) with *Strongylocentrotus lividus*.

6. Qualitative experiments testing the effect of fat solvents on unfertilized *Arbacia* eggs showed a fundamental similarity between the effects of anæsthetic concentrations of ethyl alcohol, ether, chloroform and ethyl urethane. These substances have a narrow range of high effectiveness near the point at which the cell cytolyzes.

7. Studies of the effect of the four common cations of sea water in causing polyspermic fertilization of *Arbacia* eggs showed that, when unfertilized eggs are washed in isotonic solutions of the chlorides and inseminated on return to sea water, the ions are effective in the order $Na > K > Ca > Mg$. On the other hand, insemination in isotonic chloride-sea water mixtures gave the result, $Mg \gg Na, K, Ca$, with the maximum percentage for $Mg = 71$, for Na, K and $Ca =$ ca. 5.

8. Exposure of unfertilized *Arbacia* eggs to acidified sea water over the pH range from 5.8 to 8.2 gave a maximum of polyspermy at pH 7.0. This result agrees with the work of Smith and Clowes (1924).

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THE EFFECT OF TEMPERATURE ON OVIPOSITION IN DROSOPHILA MELANOGASTER

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A study of the effect of temperature on oviposition rate in *Drosophila melanogaster* was undertaken with the hope that the egg-laying mechanism might offer suitable material for the study of growth problems in the formation of the mature egg, a process which is free from the complications of differentiation.

METHODS

To eliminate genetic differences sibling F_1 females from single-pair crosses between inbred strains of vestigial and Oregon-R wild type flies were used for the egg-laying. These inbred lines had been maintained by single-pair matings during four generations for the vestigial line, and seven generations for the Oregon-R line. Wings of the females were clipped to prevent accidental loss of flies.

Single females were isolated in shell-vials within 24 hours after eclosion from the pupa-case, and mated with one or two males. Males dying during the course of the experiment were immediately replaced.

Eggs were collected on paper ice-cream spoons (previously impregnated with bees-wax to prevent moisture absorption) containing 2 cc. of a banana-molasses food medium (100 cc. water, 20 grams banana, 5 cc. molasses, 2 grams agar) yeasted with banana-cultured yeast. Egg-laying was confined to the food surface. In cases where moisture collected on the walls of the vials, examination showed that no eggs were laid in this moisture.

Females were allowed to lay eggs for a 24-hour period on any one spoon, a record was made of the number of eggs laid, and a fresh spoon supplied for the next 24-hour period.

To detect any differences between females ovipositing at a temperature other than that at which they were bred, and those ovipositing at the respective temperatures at which they were bred, the experiments were arranged as follows:

(1) Females were bred at 16° C., 20° C., 25° C., and 28° C., and allowed to oviposit at the same temperatures.

TABLE I

Averaged data for the total eggs laid up to the end of an elapsed time. The number of females averaged is indicated by the numbers between parentheses. The group numbers at the column-heads correspond to the numbers on the curves of Fig. 1.

Days elapsed	Total eggs laid							
	All females bred at 25° C. Ovipositing at temperatures indicated					Females ovipositing at the respective temperatures at which they were bred		
	2. (5) 16° C.	4. (5) 20° C.	5. (7) 25° C.	6. (5) 25° C.	8. (4) 28° C.	1. (5) 16° C.	3. (6) 20° C	7. (6) 28° C.
1	0	0	3	8	15	0	1	9
3				80				
4		28						
5	18						24	262
6			165		464	73		
7		106						
8				478				
10	48						121	717
11			353		943			
12		221				181		
13				763				
15	119						262	1054
16			554		1258	259		
17		367						
18				977				
20	230						432	1305
21			780		1507	383		
22		529						
23				1157				
25	318						587	1477
26			1076			463		
27		711			1634			
30	411						815	1519
31			1270			511		
32		886						
35	513						1005	
36			1455			574		
37		1068						
40	591						1121	1519
41			1594			686		
42		1206						
45	629						1206	
46			1708			741		
47		1305						
50	679						1406	
51						777		
52		1422						
55	770						1550	
56						810		
57		1528						
60	816						1646	
62		1790						
64		1834						
65	856						1700	
70	875						1775	
73	901							

(2) Females were bred at 25° C., and transferred to 16° C., 20° C., and 28° C., at which temperatures their eggs were laid.

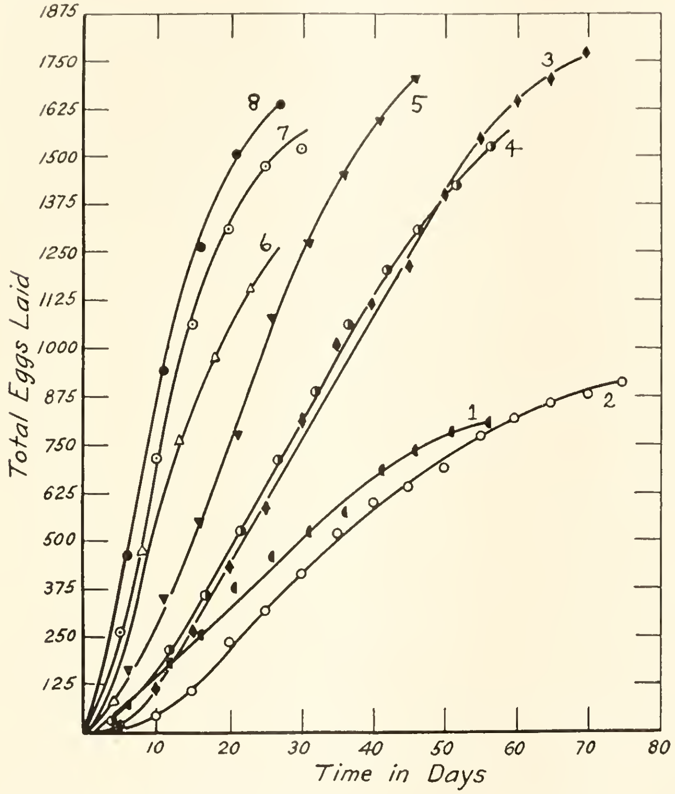

FIG. 1. Plots of the data of Table I.

1. Females bred at 16° C., and ovipositing at 16° C.
2. Females bred at 25° C., and ovipositing at 16° C.
3. Females bred at 20° C., and ovipositing at 20° C.
4. Females bred at 25° C., and ovipositing at 20° C.
5. Females bred at 25° C., and ovipositing at 25° C.
6. Females bred at 25° C., and ovipositing at 25° C.
7. Females bred at 28° C., and ovipositing at 28° C.
8. Females bred at 25° C., and ovipositing at 28° C.

Females of Curves 2, 4 and 5 are siblings. Females of Curves 6 and 8 are siblings. The counts for Curve 6 were discontinued before the end of egg-laying because of an accidental rise in the temperature of the 25° incubator. The females of Curves 1 and 2 were not followed through until the natural end of egg-laying.

The temperature for each incubator varied ± 0.5° C. from the intended value.

RESULTS

Since the rate of egg production in the fruit-fly changes rapidly with time (Shapiro, 1932), a convenient comparison of the temperature effect on egg production is obtained from a plot of the total eggs laid up to a given time. Table I gives the results for each temperature of development and egg-laying. In the table, averages of from 4–7 females (as indicated) are used. Averaging is justified because practically identical curves are obtained for each female of any one experiment.

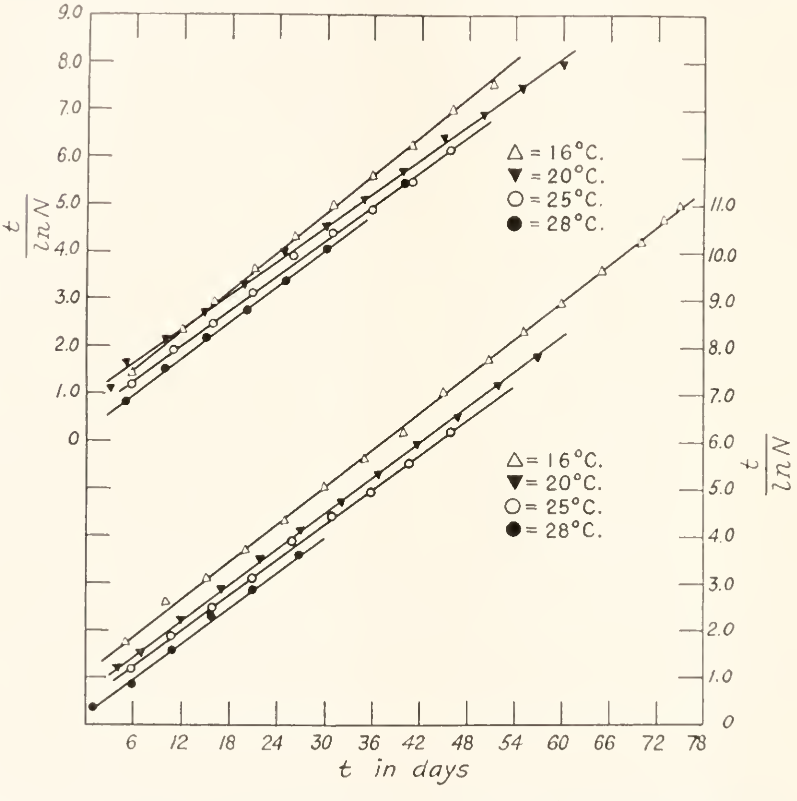

FIG. 2. Plots of the data presented in Table I, using Shapiro's equation of $t/lnN = at + b$. The curves in the upper half of the figure are for females ovipositing at the same temperatures at which they were bred. The curves in the lower half of the figure are for females that were bred at 25° C. and transferred to the other temperatures to oviposit.

The data of Table I are shown graphically in Fig. 1. Although all the curves are sigmoid in shape, their central portions may reasonably be considered linear. By disregarding the curved initial and final portions, which in all the experiments (except perhaps at 16° C.) represent very small fractions of the total number of eggs laid, we may use the slopes of the linear portions of the curves as the characteristic rates of egg-laying at the various temperatures.

The curves in Fig. 1 are grouped in pairs, representing the two different experimental set-ups at each temperature (*cf.* under "Methods" above). The two curves at each temperature can be seen not to differ significantly from each other, indicating that the rate of egg-laying is independent of the temperature at which the females were bred, and depends only on the one at which the eggs are being laid.

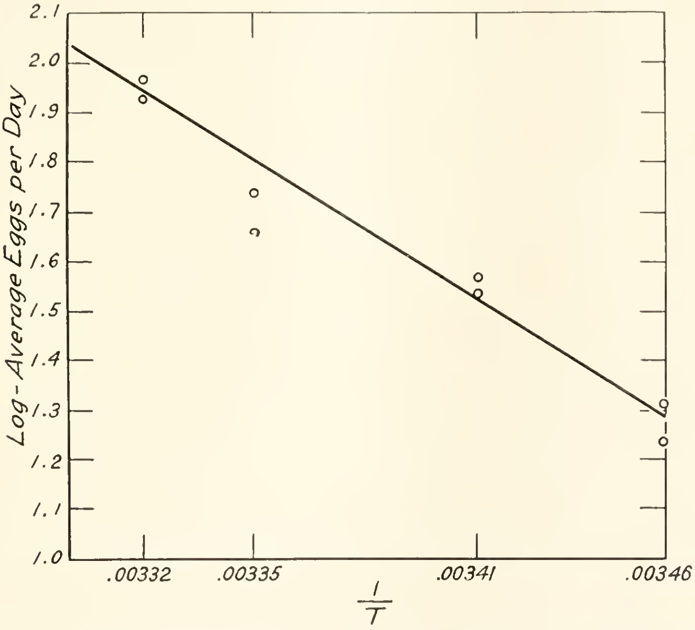

FIG. 3. Arrhenius plot of the data presented in Fig. 1. The number of eggs laid per day is calculated from the straight-line portions of the curves of Fig. 1.

The variation between the two curves (5 and 6, Fig. 1) for 25° C. is probably due to the fact that the two sets of females have different pedigrees.

Shapiro (*loc. cit.*) had shown that when the ratio t/lnN, where t is the number of elapsed days of egg-laying, and N the number of eggs laid at the end of time t, is plotted against t, a straight line is obtained. Shapiro expressed this linear relationship by the equation $t/lnN = at + b$, where a and b are constants. The same plots of t/lnN as ordinate against t as abscissa were made with our data, and the curves are shown in Fig. 2. Straight lines are obtained at each temperature, indicating that whatever relationship the equation represents remains constant when the temperature is varied.

Figure 3 represents a plot of the logarithm of the rate of egg-laying against the reciprocal of the absolute temperature. The rates of egg-laying were obtained from the slopes of the linear portions of the curves given in Fig. 1 and are expressed as eggs per day. The straight line joining the points in Fig. 3 seems to show that the Arrhenius equation applies in this case, and that the rate of egg-laying is a function of temperature in the same manner as are the rates of many chemical reactions.

Conclusions and Discussion

The regularity of the data at the different temperatures indicates that the mechanism of oviposition in *Drosophila melanogaster* may be useful for further study. The applicability of both the Shapiro and the Arrhenius equations further indicates that the underlying factors of oviposition are uniform in their action.

The rate of oviposition has been used by Dobzhansky (1935) with *D. pseudoöbscura* as an indicator of differences between geographic stocks. It has been used to measure physiological effects of genes (Alpatov, 1932) on the assumption that different Mendelian characters affect differently the developmental period of the fly.

The question arises as to what determines the total egg production throughout the life of the fly. Does it start out with a fixed amount of egg-material available for egg production, the rate of egg-laying being a measure of the rate of consumption of this material, or does the amount of available egg-material vary with the temperature under which the flies are bred, the rate of oviposition being a measure of the operation of some other factor or factors?

The data at hand are not adequate to answer the question specifically. However, examination of Fig. 1 shows that although the total numbers of eggs laid by the females at 20° C., 25° C., and 28° C., tend to approach the same asymptote, there is nevertheless a small likelihood that the curves for 16° C. will reach the asymptotes of the curves for the flies laying at the higher temperatures. Furthermore, the curves for flies bred at 25° C. and 16° C. respectively, and both laying eggs at 16° C., show identical trends as regards rate. The latter fact would indicate that the available egg-material does not enter as a factor in this case. At the higher temperatures, however, the tendency for all the curves to approach the same asymptote seems to show that the available egg-material may be a limiting factor for these flies.

The egg-laying process as a whole must involve at least four components: first, the feeding habits of the females; second, the production of secondary oöcytes; third, the accumulation of stored materials in the growing egg; and fourth, the physiological reaction of oviposition. It may be possible to separate these processes and study each of them independently.

SUMMARY

For any given temperature of egg-laying, there are no significant differences in rate of oviposition between females that have been bred at that temperature, and those that have been bred at 25° C.

The rate of egg-laying (as measured by the number of eggs laid per day) starts low, rises to a maximum, and decreases.

The rate for the middle period of egg-laying is practically constant.

The rate of egg-laying is related to temperature according to the Arrhenius equation.

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THE EFFECTS OF X-RADIATION ON THE REGENERATION OF THE HYDROID, PENNARIA TIARELLA

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INTRODUCTION

Investigations of the past decade have demonstrated that X-radiation is a valuable tool in studying the process of regeneration in various animal groups. By means of X-radiation, abnormal conditions have been produced in the tissues and cells of normally regenerating structures which result in their failure to regenerate and in this way light has been thrown on their probable function in the normal process of regeneration. Much work of this type has been done among certain phyla of the invertebrates. Among these investigations may be mentioned the work of Curtis (1928) on *Planaria;* Stone (1932 and 1933) on *Tubifex* and Turner (1934 and 1935) on *Lumbriculus.*

Only two papers have reported studies on the effects of X-radiation on regeneration processes in the cœlenterates. Curtis and Ritter (1927) state that hydranths of *Tubularia crocea* fail to regenerate after exposure to X-radiation and Zawarzin (1929) and Strelin (1929) report that regeneration in *Pelmatohydra oligactis* is inhibited by X-radiation. Strelin (1929) has made histological studies of non-regenerating X-radiated forms and states that the failure to regenerate is due to the destruction of the interstitial cells by the X-radiation.

In the present investigation studies have been made to determine (1) the effects of X-radiation on the regeneration of amputated hydranths of *Pennaria;* (2) the relative sensitivity to X-radiation of hydroid tissues as determined by the minimal dosage of X-radiation necessary to inhibit regeneration and (3) the effects of X-radiation on the regeneration of a colony of hydranths in which a portion of the colony has been exposed to X-radiation with the remaining connected portion of the colony screened from the radiation by a lead plate. The observations on these studies will be described here. It is proposed to make a histological study of the materials collected in these experiments with the hope that they can be reported at a later date.

MATERIALS AND METHODS

Colonies of the hydroid, *Pennaria tiarella,* were used in all the experiments. This form is found growing profusely on the brown alga, *Fucus*, in the vicinity of Woods Hole, Massachusetts, during the months of July and August. Fresh material was brought into the laboratory daily and only day-old material was used in the experiments. The hydranths were removed from young branches of the colony under a dissecting microscope. After radiation the branches were suspended by means of fine cotton threads in dishes of running sea water for the duration of the experiment.

The source of radiation was a Coolidge broad focus tube. The factors governing the radiations were as follows: 120 Kv.P., 5 ma., distance from target to material, 19.5 cm. The radiation was unfiltered. The output of the tube as determined by a standard ionization tube was 750 Roentgen units per minute. The only variable factor was the length of exposure and this will be mentioned in connection with the various experiments.

Normal Regeneration of Hydranths

The process of hydranth regeneration in *Pennaria* has been discussed in some detail by Gast and Godlewski (1903) and Hargitt (1904). In addition to experimental work, these investigators report histological studies on the normal regeneration of hydranths of *Pennaria* and the reader is referred to these papers for a more detailed account of the process than can be covered in this paper.

Amputated hydranths of *Pennaria* are ordinarily regenerated in 36 to 48 hours when the branches are kept suspended in running sea water at a temperature of around 22° C. Early in the investigation it was noted that *Pennaria* tended toward heteromorphic regeneration. If the pieces under observation were simply laid flat on the bottom of the dish, the cœnosarc, on emergence from the perisarc, came in contact with the dish and in most cases holdfasts were regenerated rather than hydranths. Hydranths were regenerated only at the free ends. To avoid this contact reaction the branches under observation were suspended in dishes of running sea water by fine cotton threads. In this manner all the cut ends were bathed by sea water and only hydranths were regenerated, even at the basal end of the stem. As stated above, new hydranths begin to appear 36 to 48 hours after amputation. Not all the regenerating hydranths appear in so short a time, but new hydranths continue to form on cut stems of the branch for a period of days. Colonies, on the average, were found to regenerate about 70 per cent of their lost hydranths.

If at the end of several days these regenerated hydranths are amputated, a second regeneration takes place in about the same period as that mentioned above. This process of re-amputation has been repeated for three or four successive times. New hydranths continue to regenerate.

Morphologically, the process of hydranth regeneration appears to be fairly simple. Wound closure takes place in six hours, or less, and appears to be due to an approximation of the cut edges of the cœnosarc. Regeneration processes then set in and 12 hours after amputation the first indication of a regenerating hydranth is the formation of a knob-like protuberance by the cœnosarc. This protuberance is covered by a thin layer of perisarc, which is later ruptured when the new hydranth begins to take form. This bud of cœnosarc continues to grow and tentacles appear about 24 hours after amputation as small evaginated buds on the body of the hydranth. The tentacles assume their normal length by new growth. The proboscis appears to form as an evagination from the distal end of the bud, and late in the process of regeneration the mouth opening breaks through. Regeneration is complete 36 to 48 hours after amputation.

X-radiated Branches of Pennaria

The following experiment was carried out to determine the effects of X-radiation on hydranth regeneration in *Pennaria*, and to obtain data as to the relative sensitivity of this hydroid to X-radiation, as compared with various other invertebrates on which similar studies have been made.

Small branches of *Pennaria*, possessing, on the average, twenty to twenty-five hydranths were used in all experiments. The hydranths were removed under a dissecting microscope and immediately radiated in small dishes of sea water. In the preliminary experiments dosages of 750, 1500, 3750, 7500, 10,500, 15,000 and 22,500 Roentgen units were given to various branches. The results of these experiments demonstrated that dosages of 10,500 Roentgen units, or larger, completely inhibited the regeneration of new hydranths. Dosages of less than 10,500 *r* retarded the regeneration of hydranths but were not sufficient to inhibit their formation completely. In all the later experiments, therefore, the minimal inhibiting dosage of 10,500 *r* was used.

It was found that X-radiation did not cause an immediate check on regeneration processes in radiated branches, but considerable growth of fresh tissue took place during the first twelve to eighteen hours after radiation. As a result, there was the formation of a rounded bud of fresh tissue at the cut surface of the branch, but these buds were never able to differentiate into new hydranths. Many of these radiated branches were kept under observation from 9 to 12 days after amputation without showing any evidences of differentiating into new hydranths. In some cases there was a continued growth of this new tissue, so that long stolon-like structures, several millimeters in length, were formed.

Reference to section *A* of Fig. 1 will show the extent of regeneration in radiated branches. Here it will be seen that there is a considerable proliferation of fresh tissue beyond the point of amputation but this tissue appears to be powerless to differentiate into new hydranths.

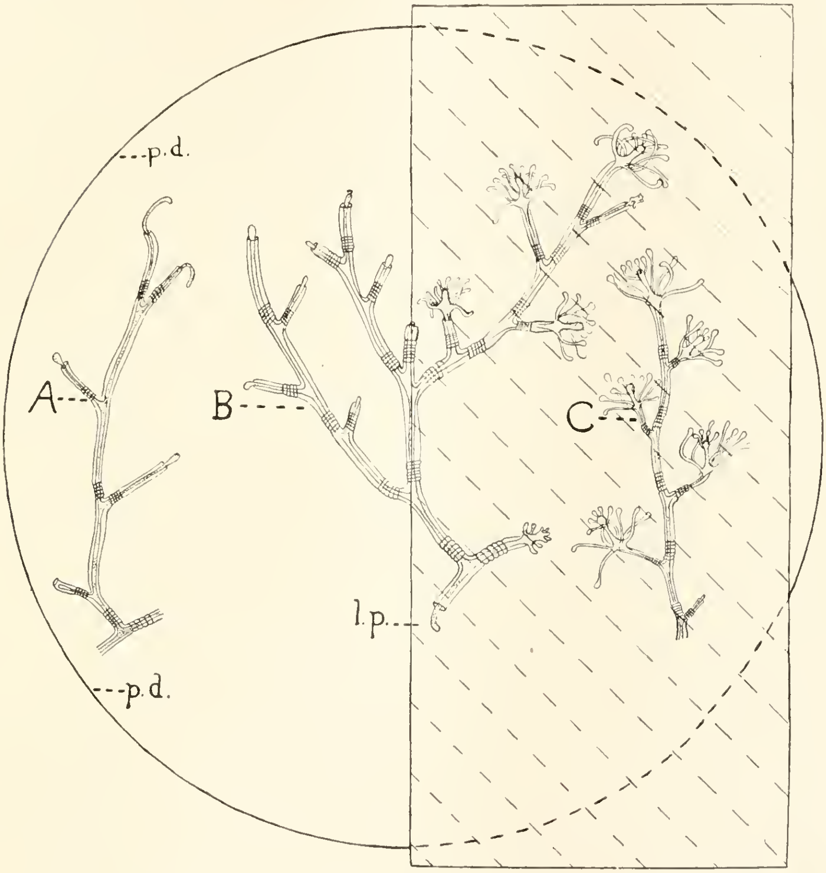

FIG. 1. Diagram of apparatus used for the radiation of a portion of a *Pennaria* colony. Colony *A* is a radiated control; colony *C* a screened control; while only a portion of colony *B* has been exposed to radiation as explained in text. *l.p.*, lead plate (cross-hatched); *p.d.*, edge of Petri dish.

If these non-regenerating branches of X-radiated *Pennaria* be re-amputated 4 to 6 days after the first amputation, the above process is repeated. A proliferation of fresh tissue takes place but there is never a formation of new hydranths.

Other experiments were carried out with branches of *Pennaria* in which radiation was made four days prior to the amputation of the hydranths. In these experiments the results were essentially the same as described above. A small proliferation of fresh tissue takes place but in no cases were hydranths ever formed.

The results of these experiments were clear-cut. Hydranths of *Pennaria tiarella* fail to regenerate when exposed to dosages of X-radiation of 10,500 Roentgen units, or more. Smaller dosages retard the regeneration of hydranths, but fail to check the process completely. The primary effect here appears to be on cellular differentiation rather than on cellular proliferation. In many cases, an adequate volume of fresh tissue is regenerated, but this tissue appears powerless to form a new hydranth.

Experiments in which a Portion of the Colony was Shielded from the Radiation

In *Pennaria*, as in other hydroids, the individual hydranths are connected by a common cœnosarc and share a common circulation by means of a cœlenteron. The question arises as to what will be the effect of X-radiation on the regenerative abilities of a colony if a portion of the colony is exposed to the radiation while the remaining portion of the colony is shielded from the radiation by a lead plate. Will toxic substances or so-called "necrohormones" be formed in the exposed portion of the colony and then pass over to retard or inhibit hydranth regeneration in the screened region? On the other hand, will the protected members of the colony exert any sort of influence on the non-regenerating X-radiated portion of the colony so as to promote regeneration in this region?

To test these possibilities the following experiment was carried out. A small Petri dish was fitted with a one-quarter-inch lead plate so that one-half of the surface of the dish was covered and the other half exposed to the radiation as shown in Fig. 1. The inner surface of the plate was bent into the shape of a flange extending to the bottom of the dish so as to prevent a scattering of the radiation under the lead plate. A piece of the central stem of a *Pennaria* colony (Fig. 1, colony *B*) with two lateral branches of approximately equal size, and from which all the hydranths had been amputated, was placed in the dish in a manner such that one branch was exposed to the radiation while the remaining connected portion was protected by the lead plate. One completely exposed colony (Fig. 1, colony *A*) and one completely screened colony (Fig. 1, colony *C*) were used as controls. The whole set-up, as described above, was given a single exposure to X-radiation of 10,500 Roentgen units.

Briefly stated, the results were as follows: The colony exposed to the radiation (colony *A*, Fig. 1) failed to regenerate its lost hydranths, even after six to eight days after radiation. The colony completely screened from the radiation (colony *C*, Fig. 1) regenerated its lost hydranths in 48 hours while the partially exposed colony (colony *B*, Fig. 1) behaved as follows: The portion of the colony protected from the radiation by the lead screen regenerated its lost hydranths in 48 hours, as rapidly and as completely as its control (colony *C*), while the exposed portion of the same colony failed to regenerate, even as long as eight days after amputation. The exposed portion behaved in all respects like the completely exposed control (colony *A*). This experiment was repeated several times with the same results.

The results obtained here demonstrate that, for the duration of the experiment, the effects of the X-radiation are purely local. The fact that screened members of the colony, although they are connected directly with non-regenerating X-radiated members of the same colony, regenerate at a normal rate tends to discount the idea that anything like a toxic material or "necrohormone" is being formed in the radiated portion of the colony which may be spread throughout the entire colony. In a like manner, there is no evidence that any regeneration-promoting substance or material is passing from the unradiated portion of the colony over to the non-regenerating branches of the radiated side. They act as if they were independent of the unradiated tissue.

Discussion

Considerable evidence is being accumulated to show that one of the chief biological effects of X-radiation is an effect on cellular differentiation. Histological studies on non-regenerating limbs of X-radiated *Amblystoma* larvæ made by Butler (1933) and Puckett (1936) show that one of the chief effects of the radiation is to prevent the differentiation of the cells of the limb stump into new limb structures. Likewise, Henshaw (1935), in his studies on the effects of X-radiation on the development of the eggs of *Drosophila*, has found that the lethal effect of X-radiation on *Drosophila* eggs is not the result of an inhibition of cell divisions, but is due to the fact that the new cells that are formed are powerless to differentiate into embryonic structures. Death results, not from a cessation of growth processes, but as a result of the loss of the powers of directive growth and organization.

Although no histological studies have been made on non-regenerating X-radiated branches of *Pennaria*, the effect here appears to be primarily on differentiation processes. In practically all cases of radiated branches of *Pennaria*, a mass of new tissue sufficient to form a new hydranth was regenerated and, in many cases, fresh tissue of several times the volume of a hydranth was formed. However, this tissue appeared to be unable to differentiate into hydranths. It seems clear that the effect of X-radiation in this case is not primarily an inhibition of growth but rather an inhibition of the ability to differentiate new structures.

The observations made here also demonstrate that the effect of X-radiation is a local one on radiated tissue. The fact that exposed portions of a colony of hydranths fail to regenerate after exposure to X-radiation, while connected portions of the same colony, screened from the radiation, regenerate at a normal rate supports the statement above. This evidence seems to show that the tissue fluids of the colony are little affected by the radiation. The various members of the hydroid colony are connected by a common cœnosarc and have a common circulation in the nature of a cœlenteron. If the tissue fluids exposed to the radiation were affected they should, in some way, affect the regeneration of the protected hydranths. This does not appear to be the case.

This latter observation agrees with and substantiates the observations of Butler (1935) in his studies on the effects of local radiations on the regeneration of the *Amblystoma* limb. He found, to mention a single experiment, that if the body of the larva be radiated with the limb screened from the radiation, then regeneration was not interfered with. On the other hand, if the body of the larva was screened and the limb exposed to the radiation, then the limb failed to regenerate if amputated. This, and a series of other experiments, showed conclusively that the effect of X-radiation was a local one on the cells of the limb, rather than on the general body fluids such as the blood or lymph. The hydroid experiments appear to confirm his belief in a purely local effect of X-radiation.

SUMMARY

Regeneration of amputated hydranths of the hydroid, *Pennaria tiarella*, has been prevented by exposure to X-radiation.

The minimal dosage of X-radiation necessary to inhibit hydranth regeneration has been determined.

The effect of X-radiation appears to be primarily on cellular differentiation, rather than on cellular proliferation. In all cases an appreciable amount of fresh tissue regenerated, but this tissue appeared powerless to differentiate into new hydranths.

X-radiation of one portion of a single colony of *Pennaria* with the remaining connected portion of the same colony protected from the

radiation by a lead screen, indicates that the effects of radiation are local. Exposed portions of the colony failed to regenerate new hydranths, while protected portions regenerated new hydranths at a normal rate.

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THE QUANTITATIVE DETERMINATION OF MITOTIC ELONGATION

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This is the first of a series of investigations dealing with a specific phase of cell division, the elongation which occurs when a cell divides. The normal elongation of the cell may be defined as follows: the spontaneous extension that ensues upon the increase in length of the diaster, and which is climaxed by the cleavage of the cell. Measurements have been made of the magnitude of this extension, and time-elongation relationships established. The present paper is concerned only with the normal process; future experiments have been planned to test the effects of various physical and chemical factors. Some of these experiments have been partially completed.

Because the process of mitotic elongation is essentially of a kinetic nature, involving changes in cell structure with respect to time, it can be studied best in the living material. It was not feasible to employ the methods of fixation and staining in order to determine the morphological changes. Nevertheless, an attempt was made to insure an adequate correlation between structure and function. This was done by selecting material that would show the cell structures most clearly in the living animal.

In this respect the egg of the common sea-urchin, *Arbacia punctulata*, possesses certain advantages. The achromatic figure (diaster) is well-defined in the living material and indicates distinctly the future axis of elongation. Since the egg is not always exactly spherical prior to the period of elongation and cleavage, the clear bipolar spindle serves to indicate, and allows one to measure, that particular diameter of the egg that will coincide with the future axis of elongation. Another advantage is to be found in the fact that the two blastomeres resulting from the first cleavage never separate entirely. This precludes any errors arising from a shifting or twisting of the blastomeres that might otherwise occur.

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[1] Opportunity is here taken to thank Dr. L. V. Heilbrunn for his valuable assistance and criticism during the course of this investigation.

The manner of procedure is exemplified by Experiment *C* of Series *A*. The particular egg used in this experiment was fertilized at 3:02 P.M., and the first measurements of the diameter were made at 3:45 P.M.—just prior to the onset of the elongation. The average value for the egg diameter was 74.9μ. Successive elongations (column 7—as computed by subtracting the initial from the final readings) were measured with a filar micrometer, and the "times" (column 4) corresponding to the readings were "tapped out" on a key connected with a kymograph on which timing intervals of 6 seconds were automatically being recorded. The maximum elongation is seen to be 7.7μ, an increase of 10.3 per cent.

SERIES *A*

Mitotic elongation of *Arbacia* eggs with fertilization membranes.

7/31/35 Fert.	Diameter *ab* of egg (scale div.) add 300		*T* sec.	Remarks	Original diameter	Successive increments	Max. Δx	Increase
	a	*b*						
								per cent
Exp. *C*			$0_t = 3{:}45$	R.T. = 23.6° C.	325.5		33.5	10.3
3:02 P.M.	22.5	46.5	P.M.	tight fert.				
	21.5	48.0		membrane				
	20.5	46.5			(=74.9 μ)		(=7.7 μ)	
	20.5	47.5						
	19.5	44.0						
	19.0	46.5	0	*		27.5		
	18.0	52.5	30			34.5		
	21.0	60.5	60			39.5		
	25.0	73.5	102	touching		48.5		
	23.0	79.5	132	fert. mem.		56.5		
	22.0	81.0	159			59.0		
	20.5	79.5	183			59.0		
	20.5	78.0	225			57.5		
	22.5	73.5	261			51.0		
	25.0	69.0	306			44.0		
	24.5	65.0	342			40.5		

One scale division = 0.23 μ.

* First appearance of the elongation.

The measurements for 27 cases are presented in Table I. The average diameter of these 27 eggs before elongation is 73.5μ, with a probable error of $\pm$ 0.224μ. The "probable error" has the usual definition, $R = \pm 0.6745\sqrt{\frac{\Sigma(V^2)}{n(n-1)}}$. The table shows that there is no correlation between the diameter of the spherical egg, and the maximum diameter that it will reach during the elongation. In other words, given two eggs of different diameters, nothing can be predicted concerning which of them will attain the greater diameter during division. The average length of the major axis of the eggs when in the state of maximum elongation is 82.0 ± 0.332μ.

The experimental temperatures (see Tables I and II) ranged from 22.2° C. to 26.0° C., a variation of 3.8° C. The variation for any one experiment was always less than 1.0° C. Some preliminary experiments conducted at low temperatures (7° C.–10° C.) indicate that this process may have a high temperature coefficient. It is indeed unfortunate that the temperature could not be more uniformly controlled; yet, in view of the large biological variation, it seems certain that the resulting source of error is quite small.

TABLE I

Mitotic elongation of *Arbacia* eggs with fertilization membranes.

Temperature	Original diameter	Max.	Δx	Increase	Temperature	Original diameter	Max.	Δx	Increase
° *C.*	μ	μ	μ	*per cent*	° *C.*	μ	μ	μ	*per cent*
23.8	73.4	81.5	8.1	11.0		71.4	77.5	6.1	8.5
24.0	75.9	84.0	8.1	10.6	23.0	71.8	81.8	10.0	13.9
23.8	71.0	79.4	8.4	11.8		72.7	86.0	13.3	18.1
23.5	72.0	81.3	9.3	12.9	22.2	72.8	81.0	8.2	11.3
23.5	74.8	83.1	8.3	11.1	23.0	78.5	87.5	9.0	11.5
23.0	73.0	82.7	9.7	13.0	23.0	74.1	82.6	8.5	11.5
23.0	74.8	79.7	4.9	6.6	22.7	72.5	81.9	9.4	12.9
23.0	71.8	80.8	9.0	12.5	22.4	76.0	84.4	8.4	11.3
23.0	74.4	80.5	6.1	8.2	23.0	74.1	87.1	13.0	17.5
23.0	74.8	80.2	5.4	7.1	22.5	70.8	76.9	6.1	8.6
23.0	72.2	78.8	6.6	9.1	23.6	74.9	82.6	7.7	10.3
	72.8	83.3	10.5	14.4	24.4	72.5	79.7	7.2	9.9
23.7	74.1	81.9	7.8	10.5	24.0	73.4	83.2	9.8	11.9
	72.8	83.6	10.8	14.8					

At R.T. of 22.2° C.–24.4° C., the mitotic elongation is 11.5 per cent; or, an egg 74.0 μ in diameter elongates to 82.5 μ.

If now, one plots "times" against "elongations" (Fig. 1), the result is two classes of curves: *A* and *B*. Type *A* is given only by those eggs whose fertilization membranes "stand off" from the egg surface. Type *B* is characterized by two maxima—due to the fact that the tight fertilization membrane impedes the elongation for a time. Cases falling in type *B* were the more numerous of the two. Indeed, the slopes of the curves, even within any one type, were quite divergent. The explanation of this is twofold: (1) the resistance of the fertilization membrane, and its distance from the egg are very variable factors; and, (2) the difficulty—especially during the first stages of the extension—of knowing if one is measuring the maximum (or true) elongation, or a lesser diameter resulting from the fact that the plane of the spindle may not be exactly parallel to the plane of the slide. As cleavage progresses, the position of the furrow and the occurrence or nonoccurrence of slipping-over of the blastomeres provide criteria of the above source of error. Whenever such a slipping-over took place, the experiment was discarded. Moreover, rotational movements of the egg—occasioned by the limiting fertilization membrane—often proved annoying.

In view of these considerations, another set of observations has been compiled for eggs whose fertilization membranes had been removed by vigorous shaking some 1–4 minutes after insemination. No deformities resulted.

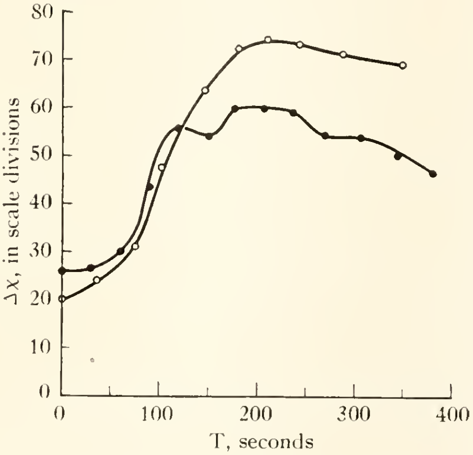

Fig. 1. Elongation of eggs having fertilization membranes. Open circles, Type *A*; solid circles, Type *B*.

A typical record is shown in Experiment *C* of Series *B*; and Table II gives the measurements for 38 cases. The average diameter of the spherical eggs is 73.5 ± 0.171 μ, and following elongation the average maximum diameter of these eggs, whose fertilization membranes have been removed, is 102.3 ± 0.434 μ. Comparing the probable errors for all eggs, independent of the experimental conditions, one notes that the probable error for eggs under conditions of maximal activity (if one may so characterize the period of elongation) is much greater than the probable error for the diameters of the eggs while still spherical. That is to say, the variations found for the period of elongation are not determined by the variations among the original diameters. Since Table II presents a greater number of cases than Table I (38 as against 27), it would be expected that the probable error of the former would be less than that of the latter. This is true for the measurements made on spherical eggs: the probable errors for the original diameters being ± 0.224 for Table I, and ± 0.171 for Table II. On the other hand, the probable error for the eggs at maximum elongation is ± 0.332 for Table I, and ± 0.434 for Table II, results that could not be expected if the laws of chance alone were operating. The explanation of this apparent anomaly lies in the fertilization membrane factor; the rigidity of the membrane cuts down the upper limit, and therefore the range of possible variation.

SERIES *B*

Mitotic elongations of *Arbacia* eggs without fertilization membranes.

8 5 35 Fert.	Diameter *ab* of egg (scale div.) add 300		*T* sec.	Remarks	Original diameter	Successive increments	Max. Δx	Increase
	a	*b*						
								per cent
Exp. *C*			$0_t = 1{:}40$	R.T. = 25.6° C.	330.0		133.5	40.5
12:58	92.0	21.0	P.M.	Removed				
P.M.	98.5	28.5		fert. mem.	(= 75.9 μ)		(= 30.7 μ)	
	81.0	14.0		1 : 01 P.M.				
	82.0	11.5						
	81.5	19.0	0	*		37.5		
	81.5	21.0	27			39.5		
	83.5	30.5	54			46.5		
	90.0	37.5	84			47.5		
	91.5	47.0	108			55.5		
	81.5	46.5	138			65.0		
	76.5	59.5	165			83.0		
	67.0	171.5	195			104.5		
	57.5	183.0	222			125.5		
	55.0	196.5	249			141.5		
	53.5	103.5	279			150.0		
	49.5	107.0	315			157.5		
	45.0	107.0	342			162.0		
	41.0	104.5	372			163.5		
	39.0	198.5	408			159.5		
	36.5	195.0	450			158.5		
	37.0	193.5	495			156.5		
	35.0	188.5	549			153.5		
	38.5	184.0	636			145.5		

One scale division = 0.23 μ.

* First appearance of the elongation.

of the elongation. As has been previously shown, not only does the fertilization membrane prevent the determination of time-elongation relationships, but it also prevents the determination of the true (maximum) extension.

In these experiments on eggs without fertilization membranes the time-elongation curves were all of one type: possessing a single maximum; and their slopes were much more in conformity with one another. The two curves in Fig. 2 show quite different maximum elongations, yet the shape of these two curves is quite similar. Indeed, if one plots on the same graph all the time-elongation curves for eggs divested of their fertilization membranes, then one finds that the width of the area covered by their ascending slopes lies within the limits of variation prescribed by the values for the maximum elongation.

TABLE II

Mitotic elongation of *Arbacia* eggs without fertilization membranes.

Temperature	Original diameter	Max.	Δx	Increase	Temperature	Original diameter	Max.	Δx	Increase
° *C.*	μ	μ	μ	*per cent*	° *C.*	μ	μ	μ	*per cent*
25.6	74.5	104.2	29.7	39.9	23.0	73.8	99.2	25.4	34.4
25.8	73.1	105.3	32.2	44.0	23.0	73.8	99.3	25.5	34.6
25.5	72.9	105.9	33.0	45.3	22.5	72.7	97.1	24.4	33.6
25.8	73.5	105.6	32.1	43.7		72.9	101.8	28.9	39.7
25.0	74.8	105.9	31.1	41.6	25.6	75.9	106.6	30.7	40.5
24.8	73.6	108.1	34.5	46.8	26.0	71.4	99.7	28.3	39.6
25.0	71.9	101.1	29.2	40.6	25.5	70.2	98.7	28.5	40.6
24.7	72.0	102.4	30.4	42.2	23.0	73.1	106.2	33.1	45.3
	72.2	96.6	24.4	33.8	23.0	73.6	107.0	33.4	45.4
24.6	75.1	105.3	30.2	40.3	22.9	73.1	96.0	22.9	30.9
25.5	73.6	104.1	30.5	41.4	23.8	74.1	106.2	32.1	43.3
25.0	73.1	100.5	27.4	37.5	23.6	75.0	106.7	31.7	42.3
24.0	71.1	93.9	22.8	32.1	23.5	74.6	108.1	33.5	44.9
24.0	75.0	101.1	26.1	34.8	23.6	76.9	103.5	26.6	34.6
23.8	74.8	104.8	30.0	40.1	23.8	75.2	106.3	32.9	42.4
23.7	71.9	97.7	25.8	35.9	23.8	73.4	104.6	31.2	42.5
23.2	73.6	99.6	26.0	35.4	24.9	73.8	102.0	28.2	38.2
23.0	72.2	96.9	24.7	34.2		74.2	101.7	27.5	37.0
23.0	73.4	94.6	21.2	28.9	25.0	72.7	102.0	29.3	40.3

At R.T. of 22.5° C.–26.0° C., the mitotic elongation is 39.4 per cent; or, an egg 74.0 μ in diameter elongates to 103.2 μ.

For convenience of analysis the curve, Fig. 2, may be divided into three parts. The first, the "incipient period of elongation," involves the slow extension that takes place during the elongation of the spindle (diaster), and the so-called "karyokinetische Streckung" (not necessarily synchronous processes). The slight rise shown during this period was not found in every case studied, but its occurrence was so frequent and definite that it has been plotted as such, and not smoothed out. This "incipient" stage is the most interesting, and yet the most difficult stage to determine. It is hoped that future experimentation will furnish a more lucid analysis.

The second stage is marked by the rapid ascent of the curve and its completion coincides with the pulling-apart of the daughter-cells (they are actually always contiguous). Some time before reaching the peak the steeply-rising curve shows a decided inflection; from this point the curve approaches the maximum much more slowly. The inflection indicates the time when the resisting influence of the hyaline-plasma membrane enters the picture. One may observe folds and wrinkles in this membrane at this period, and these probably indicate tension. Because of the fear of straying too far from the physiological picture, no attempt at removing this membrane was made—it can readily be removed by repeated washings in an isotonic (0.53M) potassium chloride solution.

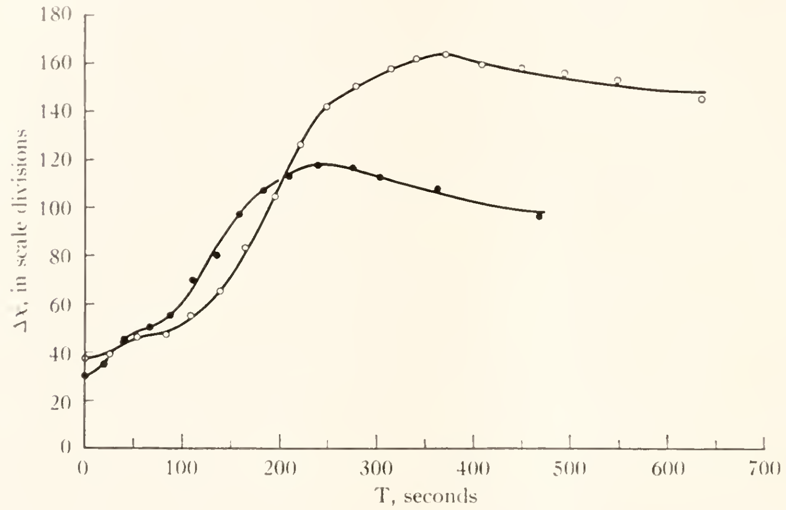

FIG. 2. Elongation of eggs after removal of fertilization membrane. Open circles, max. $\Delta x = 30.7\mu$; closed circles, max. $\Delta x = 22.9\mu$.

The curves that have been plotted of normal eggs (with and without fertilization membranes) are to be used as a standard. Experimental work on the effect of various agents on the time-elongation curves are now in progress. It is hoped that this method of attack will ultimately contribute to our knowledge of one phase of cell division; namely, the process of elongation.

The final feature of the curve is the "recovery," and is of no particular interest except to show that, at least for the length of time observed, the egg does not return to its original diameter.

SUMMARY

For the experimental temperatures (22.2° C.–26.0° C.), curves have been plotted to show the course of mitotic elongation in the *Arbacia* egg at first cleavage. For eggs possessing fertilization membranes, the elongation is 11.5 per cent (i.e., an egg 74.0 μ in diameter elongates to 82.5 μ). When eggs are divested of their fertilization membranes, the elongation is 39.4 per cent—or, an egg 74.0 μ in diameter elongates to 103.2 μ. The data obtained are being used as a basis for further experimentation.

RESPONSE TO LIGHT IN PERANEMA TRICHOPHORUM

I. Relation between Dark-Adaptation and Sensitivity to Light

S. O. MAST AND BRAINARD HAWK

(*From the Zoölogical Laboratory of the Johns Hopkins University*)

Introduction

It is well known that if photosensitive animals are subjected to darkness they become more sensitive to light, but there is much diversity of opinion concerning the relation between the time in darkness and the extent of change in sensitivity.

Hecht (1919, 1927) concludes that in *Mya arenaria*, *Pholas dactylus*, *Ciona intestinalis* and *Homo sapiens*, as the time in darkness increases, the sensitivity increases at first rapidly, then slowly, until it reaches a maximum, and then remains constant. Similar conclusions were reached by Obreshkove (1921) for *Rana clamitans*, Folger (1925) for *Amœba proteus*, Crozier and Wolf for *Agriolimax campestris*, and Hartline for *Limulus polyphemus*.

Dolley (1929) found in observations on *Eristalis tenax*, that as the time in darkness increases, the sensitivity increases to a maximum in about sixty minutes, then remains nearly constant for two hours, then decreases rapidly to a point lower than that which obtains for light-adapted specimens.

Mast (1932) maintains that in *Volvox* the relation between sensitivity and dark-adaptation depends upon the extent of preceding illumination. He says that if colonies have been in strong light one or two hours before they are subjected to darkness the sensitivity increases to maximum in approximately sixteen minutes and then decreases, but that if they have been in strong light nine hours the reverse obtains, i.e. it decreases to minimum in approximately sixteen minutes and then increases.

Material and Methods

The *Peranemæ* used in the observations described below were cultured in the laboratory. About 250 cc. pond water and 2 grams of beef suet were put into each of several finger bowls. The finger bowls were then covered and left one week or more after which a few *Peranemæ* were added to each. The *Peranemæ* in these cultures became very abundant in a week and continued in good condition for a month or more.

The observations were made in a basement darkroom in which the temperature was 21 ± $\frac{1}{2}$° C. A 1000-watt, 120-volt projection lamp was mounted in a ventilating shaft in front of an opening 2 cm. square. This lamp was in a circuit maintained at 110 volts, by means of variable resistance. A small ruby lamp was mounted in front of the microscope a little above the beam of light from the 1000-watt lamp. The light from the two lamps was, by means of a front surface silvered mirror, reflected up through a compound microscope which had no substage condenser. The intensity of light from the 1000-watt lamp was, on the stage of the microscope, 2,000 meter candles. The red light from the ruby lamp was very weak.

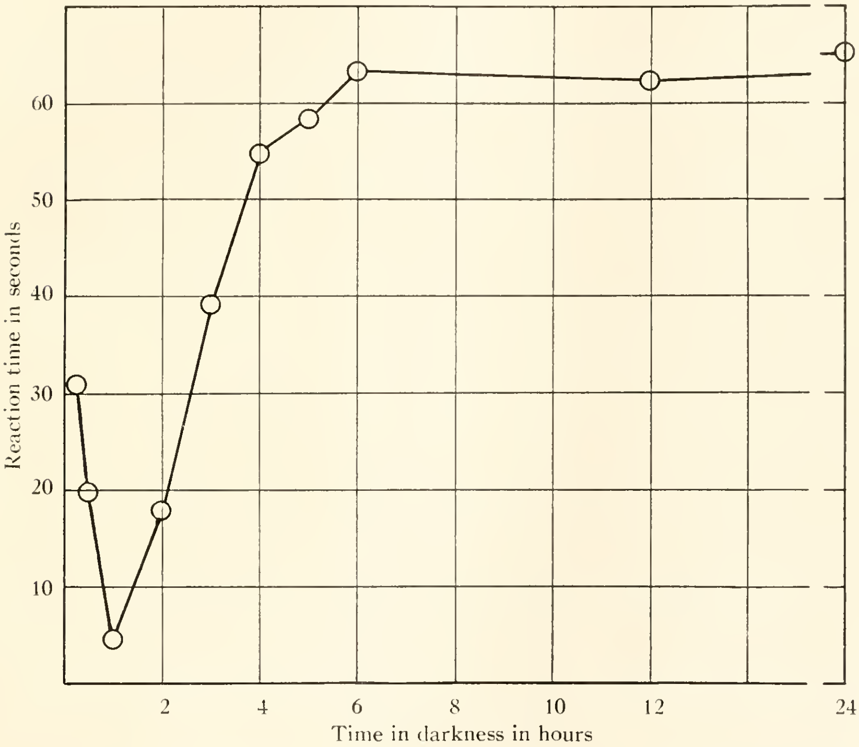

FIG. 1. Graph showing the effect of dark-adaptation on sensitivity to light in *Peranema trichophorum*. Each point on the curve, except the last two, represents the average reaction-time for 15 to 17 tests. The last two represent the average for 3 tests each (see Table I).

A shutter and a 4 cm. glass heat screen, filled with water, were mounted in the beam of light near the shaft. The shutter contained a sliding panel with two opaque plates and an opening 3 cm. square between them. This panel was mounted in a frame in such a way that it could be readily raised and released by means of cords which extended to the microscope. It had a 50-gram weight attached to the bottom to increase the rate of fall.

TABLE I

Relation between dark-adaptation and sensitivity to light in *Peranema trichophorum*. Different individuals were used for each observation. The intensity of the light used was 2,000 meter candles.

Series	Time in darkness in hours									
	1/4	1/2	1	2	3	4	5	6	12	24
	Reaction-time in seconds									
1	33.5	18.0	5.1	17.5	31.9	51.9	66.1			
	38.0	19.4	3.6	18.4	29.1	61.1	71.7	51.2		
	28.8	17.2	4.2	19.2	31.6	47.8	74.9	67.6		
	38.6	19.7	3.1	29.1	27.4	53.9	52.3	64.1		
2	29.1	23.2	2.4	11.4	42.4	44.2	54.6	77.2		
	31.7	17.6	4.1	17.6	37.1	49.8	46.8	68.2		
	26.4	14.1	4.8	21.9	29.2			59.4		
	27.2	22.8	3.9	15.4						
3	38.4	22.1	6.1	19.8	54.1	58.6	41.6	59.4		
	29.2	20.1	3.9	11.7	44.3	48.1	62.8	67.1		
	32.6	14.6	5.4	15.4		68.0	74.0	78.4		
4	32.1	19.7	4.8	22.7	59.6	49.2	51.6	54.3		
	26.4	25.2	3.1	15.9	39.7	58.3	67.4	72.3		
	30.2	17.4	5.1	18.1	41.2	71.1	62.1	64.1		
5	31.1	21.6	7.1	18.9	34.6	42.6	56.1	61.1	69.3	59.3
	26.2	17.4	3.9	23.4	32.1	59.7	47.0	60.4	57.8	72.8
	29.1	24.1	5.4	17.1	34.4	68.2	64.3	48.3	60.6	64.3
Total avg.	30.95	19.8	4.54	17.94	39.19	55.09	58.58	63.46	62.56	65.47

The observations were made as follows: Some fluid was taken from a flourishing *Peranema* culture and filtered; *Peranemæ* were then taken from this culture and one was mounted on each of 24 to 30 slides, in a drop of the filtered fluid under a cover-glass supported with a vaseline ring. These slides were now taken to the darkroom and put into a light-tight moist chamber. After several minutes the 1000-

watt and the ruby lamps were turned on, the panel raised (so as to intercept the light from the 1000-watt lamp), one of the slides transferred to the stage of the microscope and the *Peranema* brought to focus in the red light. The *Peranema* was then suddenly exposed to light from the 1000-watt lamp by releasing the panel and the time required to induce a response measured. This was repeated with each of the other *Peranemæ* on the slides after having been in darkness different periods of time. These specimens were then discarded. This whole process was repeated four times. In four of the five series of observations the time in darkness extended to six hours and in one to twenty-four hours.

The response in *Peranema* to sudden increase in luminous intensity is marked and precise. It consists of sharp deflection of the anterior end of an "all-or-none" type. This made it possible to measure the reaction-time fairly accurately.

The cultures were kept in a north window. The experiments were made in April and May and each one was started about 2 o'clock in the afternoon. The *Peranemæ* used in all the experiments were therefore thoroughly adapted to fairly strong diffuse daylight when they were taken to the darkroom.

RESULTS

The results obtained are presented in Table I and Fig. 1.

Table I shows that there was remarkably little variation in the reaction observed in the different individuals used. The probable error was only 0.18 to 3.88. This table and Fig. 1 show that as the time in darkness increased, the reaction-time decreased to a minimum in about one hour, then increased to maximum in about three hours and then remained nearly constant for at least twenty hours.

SUMMARY

If light-adapted specimens of *Peranema trichophorum* are subjected to darkness, the sensitivity to light increases rapidly to maximum (as the time in darkness increases), then decreases slowly to minimum after which it remains nearly constant. The sensitivity reaches maximum after about one hour in darkness and minimum after about four hours.

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PULSATION OF BLOOD VESSELS IN OYSTERS, OSTREA LURIDA AND O. GIGAS[1]

A. E. HOPKINS

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Blood vessels are recognized to undergo frequent changes in diameter as a result of activity of muscle fibres in their walls. The capillaries of vertebrates, although of relatively simple structure, are highly variable, due to the reactions of "Rouget cells" along their walls (Krogh, 1922). Similar cells were described by Parker (1923) in an annelid, *Nereis*. In this species Federighi (1927, 1928) described the structure of the pulsating dorsal blood vessel and "hearts" as being activated by cells ranging from simple stellate form to typical smooth muscle cells.

In lamellibranchs, however, few blood vessels are found which have the definite and characteristic walls possessed by annelids as well as vertebrates. Most of the circulatory system consists of relatively indefinite spaces within the tissues. Even an endothelium appears to be lacking save in such vessels as those in the gill filaments (Kellogg, 1890).

Description was recently published (Hopkins 1934*a*, 1934*b*) of a pair of large peripheral vessels, or "accessory hearts," in the walls of the mantle which pulsate rhythmically. This finding is of interest in view of the general absence of definite walls in the peripheral vessels. The present study was made for the purpose of throwing some light on both the structure of pulsating vessels and their mode of activity.

At the time of writing this paper a publication (Elsey, 1935) has just appeared which interprets the activity of the "accessory hearts" as pumping blood from the gills and circumpallial arteries into the auricles. Elsey stated (p. 149), "Although it has not been possible to trace the direction of the blood in the pulsating organs, the fact that they are, through their connection with the pallial veins, in communication with the auricles, leaves scarcely any doubt that part of the blood from the gills and circumpallial blood vessels passes through them toward the heart and that subsequently this blood with that of the main pallial vein is forced by contractions of the accessory pulsating organs into the auricles."

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[1] Published by permission of the Commissioner of Fisheries.

Pulsating Vessels in Ostrea lurida

Observation of the activity of blood vessels in the mantle of the intact oyster is difficult because of the heavily calcified shell. The accessory hearts were made visible by sawing away portions of one valve, allowing the mantle to curl back over the cut edge. However, this method did not permit microscopic examination by transmitted light whereby it would be possible to observe the movement of blood corpuscles.

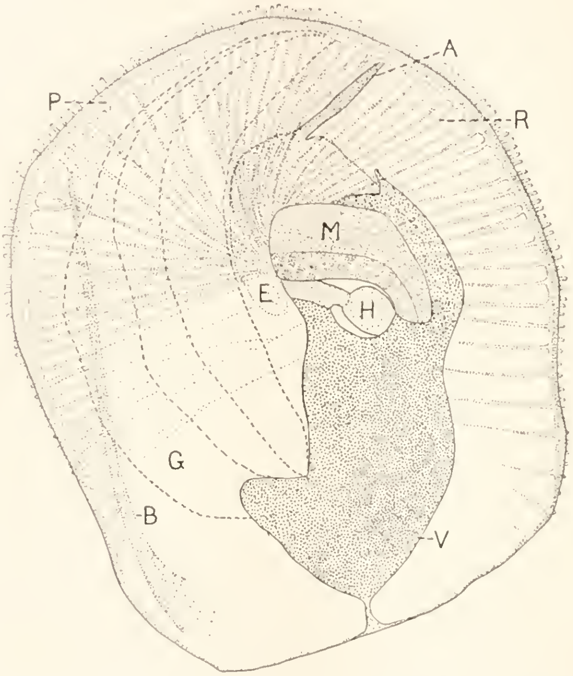


Fig. 1. Right mantle of oyster about 1 cm. in diameter as seen by transmitted light. *A*, accessory heart; *B*, waste canal; *E*, excretory organ; *G*, outline of underlying gills; *H*, heart; *M*, adductor muscle; *P*, circumpallial sinus; *R*, radial vessels; *V*, visceral mass.

In order to make such a study, glass plates were placed upon oyster grounds near Olympia, Washington, and the oysters which caught on them were allowed to grow to diameters ranging up to about two centimeters. A plate could be placed directly upon the stage of the microscope, the upper (right) valve of the specimen scraped clean, and the tissues observed uninjured by means of a bright transmitted light. Oysters about one centimeter in diameter were most satisfactory, for at this size the valves are not sufficiently calcified to render them opaque.

At the beginning of the study of such material it was a great surprise to the observer to see not only the actively pulsating heart and accessory hearts, but also a great number of smaller vessels pulsating with a slow but pronounced rhythmicity. One gets the impression that the entire mantle is involved, so numerous are the vessels and so distinct the waves of pulsation along them. Elsey (1935, p. 148) mentioned that, "Observation of young oysters from 1 to 2 millimeters in diameter showed that many of the radial blood vessels at this early stage pulsate in a manner quite similar to the accessory pulsating organs of the adult."

A drawing (Fig. 1) is reproduced to show the vessels of the right side of a specimen about one centimeter in diameter. At this size the general anatomy is that of the adult oyster. The heart lies just anterior to the adductor muscle. In the wall of the mantle the excretory organ (organ of Bojanus) adjoins the posterior and ventral borders of the muscle and extends forward under the pericardium. The accessory heart proceeds dorsally from the posterior portion of this organ. Radiating from the region of the adductor muscle and excretory organ, and anteriorly from the visceral mass, are a great many well-defined bands (*R*). Each one of these consists of a bundle of muscle fibres, the retractor muscles of the mantle. Each bundle of fibres appears to be associated with and partially surrounded by a blood vessel which pulsates rhythmically (Fig. 2).

Another active vessel is associated with the waste canal (*B*), which runs as a prominent band from near the labial palps at the anterior end to a point near the ventro-posterior edge of the mantle, where it becomes continuous with one of the radial vessels. The epithelium of the waste canal is ciliated, the cilia beating posteriorly while those of the adjoining mantle surface beat toward this band. The waste canal is both structurally and functionally identical to similar organs found particularly in lamellibranchs with well-developed siphons (Kellogg, 1915), though in the case of *Ostrea lurida* the edges are not as prominently erect. In *O. gigas*, the structure seems to have disappeared entirely. Associated with the waste canal throughout most of its length, except near the anterior end, is a blood vessel which may be observed to pulsate.

All of these vessels, including the accessory hearts, open peripherally into the vessel which runs around the margin of the mantle lobe. In some species such as *O. angulata* (Leenhardt, 1926) and *O. gigas* (Hopkins, 1934) this vessel may correctly be called the pallial or circumpallial artery, for the heart pumps blood directly into it. In *O. lurida*, however, it would appear more correct to call it the circumpallial sinus. Although a great many attempts were made to inject a colored solution into this vessel by way of the ventricle, as is readily possible with *O. gigas*, no success was had. Also, branches of the aorta spread fan-like into the mantle tissues of *O. lurida* though not of *O. gigas*. In the latter species the circumpallial vessel is very large and prominent anteriorly and relatively small along the posterior border. In fact, even in the shells of large specimens the imprint of this structure may be seen as a shallow groove. In the former species the vessel is prominent posteriorly and seems almost to disappear at the anterior end.

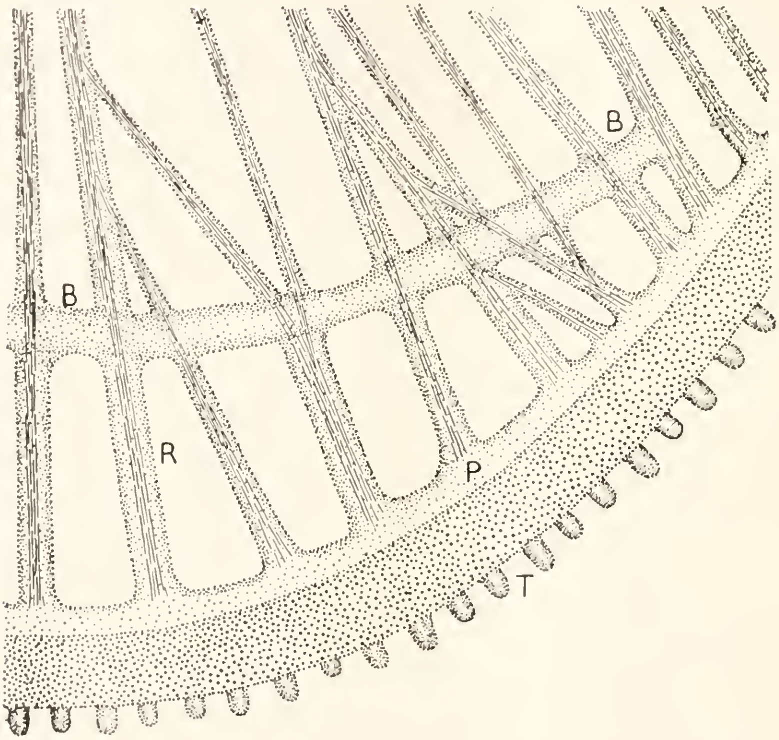


FIG. 2. Drawing of portion of inner surface of left mantle showing radial bands of muscle fibres associated with radial vessels (*R*). *B*, posterior end of waste canal; *P*, circumpallial sinus; *T*, tentacles.

Whether the circumpallial sinus also pulsates independently is not clear, though its diameter changes tremendously as waves of pulsation pass over the radial vessels. It appears probable that this vessel also is active since it is similar in structure to the others as well as continuous with them.

In a previous work (Hopkins, 1934) in which the accessory hearts were described, it was stated that the wave of pulsation originates at the central end, where the accessory heart joins the kidney, and travels slowly distally. At that time the observer was unable to watch the actual movement of blood corpuscles. The small specimens of *O. lurida*, attached to glass, gave opportunity to determine the direction of flow of blood during pulsation.

As indicated in Fig. 1, the accessory heart is directly continuous with the tissues of the excretory organ. The latter consists of a sinus in which the nephridial tubules are suspended. Blood corpuscles may be seen moving about in the blood spaces of the excretory organ and into the accessory heart. A wave of contraction starts apparently within the excretory organ itself and extends outward along the accessory heart forcing the blood rapidly ahead of it. As relaxation occurs corpuscles may be seen entering the accessory heart from both distal and proximal ends, indicating the absence of an effective system of valves. In some cases relaxation involves a definite flow of blood back into the excretory organ, though such does not seem to be typical. This reversed direction of flow is probably traceable to pressure in the circumpallial sinus created by discharge into it of other vessels.

The activity of the radial vessels is essentially similar to that of the accessory hearts in that the wave of constriction and the most rapid flow of blood originate centrally and travel to the periphery of the mantle. Absence of valves permits considerable return of blood on relaxation.

Because of the difficulty of seeing fine details, the origin of blood entering the radial vessels is still not clear. At least some of those in the posterior region definitely receive blood from the excretory organ, as in the case of the accessory heart. Prominent corpuscles have frequently been observed to enter the excretory organ on relaxation of the accessory heart and proceed distally through a neighboring radial vessel. However, the tissues of the whole region of the mantle in the vicinity of the adductor muscle and excretory organ consist of a network of irregular blood spaces which are continuous with the radial vessels and probably also with the excretory organ.

While blood in these pulsating vessels may be observed to move back and forth, according to the phase of pulsation, non-pulsating pallial veins may be observed in the same region which are returning blood from the mantle directly to the auricle. Blood in these veins moves only in one direction, in a periodic manner due to activity of the heart. These veins are clearly defined and entirely different from the pulsating vessels, and they are adjacent to the outer surface of the mantle next to the shell, while the others are near the inner surface.

Activity of the longitudinal vessel which underlies the waste canal (Figs. 1 and 2, *B*) begins anteriorly and travels as a wave of pulsation which appears to be coördinated with pulsation of radial vessels crossing it and which in at least some cases are continuous with it. Blood corpuscles have been observed to flow from the waste canal vessel into radial vessels, though there is some flow in the reverse direction during relaxation. Anteriorly many of the radial vessels appear to originate in the waste canal vessel, though it is possible that they may be in connection with more central blood spaces which are not prominent enough to be seen.

The activity of all of the pulsating vessels appears to be loosely coördinated. They do not all pulsate synchronously, but rather as if a progressive wave slowly involves them. It has not been possible to trace the origin of a wave of transmission from vessel to vessel, and it is unknown whether the pulsation originates centrally or within the vessels themselves. Its very slow rate is suggestive of the activity of smooth muscle. The rate of pulsation is approximately the same as that of the accessory heart, and probably identical with it since the latter appears to be merely the most prominent of such vessels.

The circumpallial sinus is observed with some difficulty because of the pigmentation of this portion of the mantle. Whether it actually pulsates is therefore questionable. Blood may be observed to flow for a few seconds in one direction and then reverse itself, depending upon the phase of pulsation of the radial vessels. The pulse to be observed is that of the radial vessels and accessory heart and is much slower than that of the systemic heart.

The observations described above were made primarily upon small oysters, or "spat." However, these active vessels are not confined to the small specimens. They have been observed in adult oysters as well, though in this case the movements of blood corpuscles were not readily observable. By removing the right valve of an adult specimen and allowing the right mantle lobe to curl up out of the way, it was possible to observe the activity of vessels in the left lobe of the mantle.

The radial vessels are clearly associated with the radial retractor muscle bundles of the mantle. In Fig. 2 is a drawing of a portion of the mantle showing the posterior end of the waste canal and the radial muscle bundles and vessels. It will be noted that the muscle bundles are covered with and partly surrounded by the blood vessels. These structures stand up as pronounced ridges on the inner mantle surface, particularly when the vessels are in the expanded condition. The vessels are larger distally than centrally and receive numerous small vessels, not shown in the figure.

INDEPENDENT ACTIVITY OF ACCESSORY HEARTS

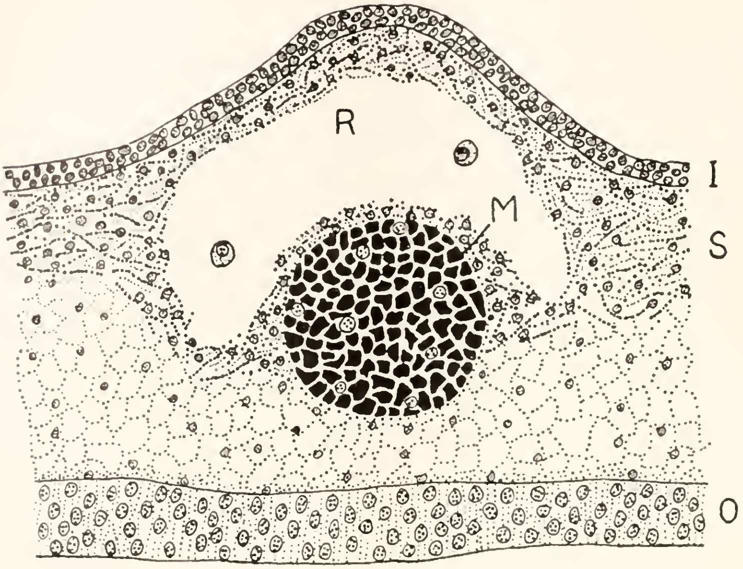
The question arose as to whether the rhythmicity of such peripheral vessels as accessory hearts originates centrally or within the organs themselves. It was found in a previous work (Hopkins, 1934) that the rate of pulsation of the accessory hearts in *O. gigas* is roughly about one-third that of the heart. It is reasonable to assume that the rate of activity of such distal vessels would be dependent upon the rate at which they fill with blood, since stretching smooth muscle characteristically stimulates contraction. However, it would be interesting to determine if pulsation occurs at all when the organs are separated from the systemic circulation.

For this purpose use was made of large Japanese oysters (*O. gigas*), in which the accessory hearts are 30 to 40 millimeters long. A specimen was removed from its shell, and a portion of the mantle wall of the cloacal chamber, including an accessory heart and a portion of the distal end of the gills, was excised. This was kept in running sea water. When the mantle was cut all of the blood vessels as well as the retractor muscles became contracted. The accessory heart, which had a diameter when expanded of 3 or 4 millimeters, contracted to about one millimeter in thickness.

The specimen remained in the contracted state for from 36 to 48 hours before the accessory heart showed any indications of relaxation. However, as soon as this organ began to expand one could see feeble pulsating movements occasionally at the distal, uninjured end. Thereafter it progressively expanded, within about a day, to what appeared to be the normal diameter, and rhythmic pulsation became more pronounced and finally included the entire vessel.

Excised portions of mantle like that just described were prepared in several instances. They lived well as long as they were kept in sediment-free, running sea water. They have been kept in good condition for periods as long as 30 days. Death in all cases occurred as a result either of stoppage of the water supply or the presence of a quantity of sediment which permitted bacteria to get a foothold. The specimens were of interest in that they exhibited apparently normal activity. The tentacles frequently moved and were sensitive to stimulation by chemicals and touch, and even reacted to a decrease in the intensity of light. The cilia of both mantle and gills were active and the latter pumped water. Due to the action of cilia, specimens moved about all over the dish in which they were kept. On the outer surface of the mantle secretion of shell was well begun.

The accessory heart pulsated with a slow rhythm and at a rate comparable to that of such organs in the intact oyster (Hopkins, 1934).



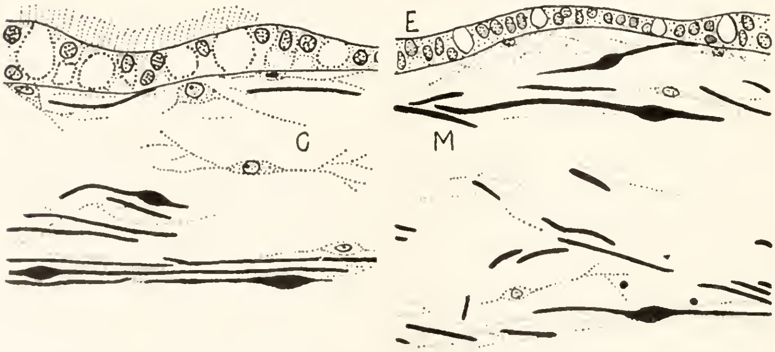


FIG. 3. Semi-diagrammatic drawing of portion of cross-section of mantle of *Ostrea lurida*, showing radial vessel (*R*) associated with radial band of muscle fibres (*M*). *I*, epithelium of inner, and *O*, of outer surfaces of mantle; *S*, sub-epithelial matrix of connective tissue and smooth muscle fibres.

FIG. 4. Two portions of wall of expanded accessory heart of *O. gigas*. *E*, epithelium; *C*, connective tissue cell; *M*, smooth muscle cells.

After the excised portion had become well adapted, the wave of pulsation was obviously from the central to the distal end. The rate of transmission of the wave as measured over a length of about 30 millimeters was generally from 4 to 8 millimeters per second at 12° to 15° C., though actual contraction was not completed for 3 to 4 seconds more. Such a slow rate of conduction compares favorably with the rate of 5 to 20 millimeters per second given by Clark (1927) for conduction by the plain muscle of the frog's stomach at 15° C.

Structure of Pulsating Vessels of Mantle

The high degree of active pulsation exhibited by the numerous blood vessels in the walls of the mantle is surprising in view of the indefinite nature of most peripheral blood channels in lamellibranchs. It is generally known that the main arteries break up into blood spaces within the tissues. After observing the activity of the mantle vessels, it was thought likely that microscopic study would show them to be definitely walled, regular vessels, similar to the "hearts" of *Nereis*, with an endothelium and circular muscle fibres.

Serial sections were made of entire Olympia oysters (*O. lurida*) and of one of the isolated portions of mantle described above. Sections were stained either with Ehrlich's hæmatoxylin or Heidenhain's iron hæmatoxylin, with eosin as counterstain.

The epithelium of the inner mantle surface is simple, consisting of a single layer of cells which vary from very thin to semi-columnar. Goblet-cell mucus glands are abundant and some of the cells, chiefly the longer type, are ciliated. Underlying the epithelium is a matrix of connective tissue in which numerous smooth muscle cells may be seen (Fig. 3).

The sub-epithelial tissue appears as a loose network in which open spaces of all sizes and shapes with indefinite boundaries occur. These are the blood spaces or "vessels." Normally fixation of a specimen results in the contraction of many of these spaces, in which case one can see no histological indication of what was, in the expanded condition, possibly a prominent radial vessel. Most of the sections across the radial vessels (Figs. 2 and 3) show well the bundles of radial muscle fibres, but only occasionally is the cavity of the blood space thoroughly open. Kellogg (1890) figured the accessory hearts in cross-section but described them as ridges, probably because the contraction resulting from fixation left no remaining cavity. Yet at least the larger of the vessels are obviously definite in location. The spaces may not be seen when in a state of contraction only because of the absence of well-formed walls.

Sections of the accessory heart (Fig. 4) of one of the isolated pieces of mantle, fixed in a state of complete expansion, show more clearly the location and mode of functioning of the sub-epithelial smooth muscle fibres. The cavity of the vessel appears to be in the middle of the network of tissue so that when expanded the outer wall is suggestive of a well-defined structure. The muscle fibres appear to run in all directions. In certain cases it has been thought that an endothelium was present, but this was probably only the connective tissue fibres stretched by expansion of the vessel. Frequently one may see either muscle fibres or strands of connective tissue extending across the cavity of an expanded vessel. Elsey (1935) considers the sub-epithelial muscle fibres of the accessory hearts to constitute circular muscle. This may be correct, but it is possible that such an appearance may be due only to the expansion of the vessels and consequent stretching of fibres.

Discussion

Activity of blood vessels is generally associated with a definitely walled structure of a specific nature, as in the typical arteries, veins, and capillaries. Lacking such structure and consisting only of irregular spaces within the tissues, the blood channels of the oyster are subject to changes in diameter of a rhythmic nature which result in producing a flow of blood within the tissues of the mantle. It would seem hardly probable that such pronounced rhythmic activity of peripheral vessels as that described above is a peculiarity of oysters alone, or even of lamellibranchs.

The inner surfaces of the mantle of lamellibranchs, with their rich supply of blood spaces, are commonly considered to constitute the principle organ of respiration, while the gills function largely in the feeding activity and have a relatively slight blood supply. On observing under proper conditions the flow of blood through the spaces in the tissues of the mantle of *Ostrea lurida* as influenced by rhythmic contractions of the relatively unformed and indefinite vessels, one readily agrees that the mantle may constitute the primary respiratory organ. Although it would be difficult to observe, it is considered probable that blood spaces throughout the body of the oyster are similarly active, for smooth muscle fibres may frequently be seen near them.

It was suggested (Hopkins, 1934) that the accessory hearts of the oyster may be homologous to the "gill hearts" of cephalopods, and it may be that this is the correct interpretation from the evolutionary point of view. One may think of a particular pair of the active radial vessels becoming larger as they take over more and more of the function of supplying blood to the organs of respiration. In *O. gigas* the radial vessels, with the exception of the accessory hearts, are less prominent or, at least less readily observed to pulsate, than in *O. lurida*. In the former species the heart pumps blood directly into the pallial artery into which the accessory hearts also empty, thereby possibly functioning to supply the tissues of the mantle with the blood required for respiration. This blood supply is provided in the latter species primarily by the pulsating radial vessels.

Elsey's (1935) interpretation of the function of the accessory hearts in *O. gigas* is that they pump blood into the auricles, rather than from the blood spaces of the excretory organs into the circumpallial artery, as previously described (Hopkins, 1934). He gave no evidence in support of the hypothesis save that proximally they communicate with pallial veins. Although it must be admitted that the function of these organs is not yet entirely clear, it is difficult to understand why such large, active structures should be developed in an organism to pump blood from a large artery (the circumpallial) directly back to the auricles. However, he did not describe either the direction of the impulse of contraction or the blood corpuscles in motion.

The mechanism whereby the gills of most lamellibranchs receive an adequate blood supply is generally not clear. Grave (1909) described the mode of circulation through the gill lamellæ of *Atrina* in detail. Blood enters the reflexed lamellæ from a large vein originating in the kidney and leaves through the direct lamellæ. Since the blood from the ventricle passes first through the tissues of the viscera before being collected in the spaces of the kidney, it is probable that a pulsation of the afferent veins of the gills would be necessary to force the blood through the small vessels of the gill filaments.

Similarly, in *Mytilus* the arrangement of afferent and efferent veins of the gills would appear to imply the feeble circulation which Field (1922) ascribes to them unless the afferent vessels are capable of pulsation. Kellogg (1890) observed the blood of *Arca* to flow back and forth through individual gill filaments. Corpuscles were observed to move outward for about half a minute and then reverse their direction.

These examples are selected from the extensive literature on the subject merely to suggest that an understanding of the possible pulsating activity of afferent branchial veins might result in clearer ideas of the physiology of lamellibranch gills. Although the accessory hearts of the oyster may pump some blood into the gills (Hopkins, 1934), it appears that these organs are not the chief source of the branchial circulation, as pointed out by Elsey (1935). The blood vessels of the gills are directly continuous with the large veins to which they are attached and which return blood from the visceral mass to the excretory organs. These large veins are difficult to observe in the intact oyster, but they have frequently been seen to change their diameter slowly, at times with what appeared to be a sluggish rhythmicity, suggesting that a back and forth movement of blood through the filaments might result. Observations by Elsey (1935) indicate that blood vessels in the principle filaments of the gills react to stimulation with a consequent reduction in diameter.

The difficulty in detecting pulsation of peripheral blood vessels may well be due to their very slow rate. A pulsating activity with a periodicity of several minutes would not be identified as rhythmic, though it might be as much so as the activity of the heart itself. In several instances series of stop-watch readings have been taken of the slow expansion and contraction of the large medial vein to which the inner gills are attached. Although the exact time of beginning and ending of any phase could not be determined, the measurements for a complete contraction at 15° C. vary between 100 and 150 seconds. Whether this activity constitutes rhythmicity is not established, though it appears probable.

Summary

1. In the mantle of *Ostrea lurida* is a series of slowly pulsating blood vessels.

2. The many radial vessels are associated with the bundles of retractor muscle fibres of the mantle. The "accessory hearts" appear to be similar to these, though much larger.

3. The waste canal, a band of tissue running anteroposteriorly in the ventral wall of each mantle lobe and covered with cilia beating posteriorly, overlies a pulsating vessel which crosses and is continuous with many radial vessels.

4. All of the pulsating vessels communicate distally with the circumpallial sinus, which appears in this species not to be directly connected with the heart.

5. The vessels described pulsate at a slow rate and their activity is loosely coördinated.

6. The wave of contraction of the radial vessels originates centrally and travels slowly distally, forcing the blood outward into the circumpallial sinus. The pulsation of the vessel underlying the waste canal appears to begin at the anterior end.

7. The absence of effective valves is shown by the reversal of direction of flow of the blood as relaxation takes place.

8. The blood supply of the radial vessels appears to come from the blood spaces of the excretory organs and nearby tissues.

9. Isolated portions of the mantle of *O. gigas*, including an accessory heart, live for weeks, the accessory heart pulsating at approximately its normal rate. The rate of transmission of the impulse of contraction is slow, 4 to 8 mm. per second. The activity is compared to that of vertebrate smooth muscle.

10. The pulsating vessels appear to be indefinite spaces within the sub-epithelial matrix of connective tissue and smooth muscle, and an endothelium and other well-defined structures characteristic of formed blood vessels may be lacking.

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THE PRECISE CONTROL OF GROWTH IN A PURE CULTURE OF A CILIATE, GLAUCOMA PYRIFORMIS[1]

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INTRODUCTION

Biologists have long demanded cultures of animal cells for a wide variety of investigations. In the past the usefulness of protozoans for biological research has been greatly limited by the necessity of bacteria for food. None of the larger forms has been grown in the absence of other life, i.e., pure culture. However, a small number which can be handled readily as individuals when magnified only a few diameters under the dissecting binocular microscope has been recently obtained in pure culture, and some of these have already proved useful.

The present paper describes a medium which supports extremely vigorous growth of *Glaucoma* in pure culture. It presents a quantitative analysis of the characteristics of growth of *Glaucoma*, and shows that the control engendered by the culture method permits duplication of similar experiments as frequently as desired.

IDENTIFICATION

The holotrichous ciliate *Glaucoma pyriformis* is a facultative parasite and is capable of growth under strictly anærobic conditions.

Figure 1 contrasts *Glaucoma* (*a*) with *Colpidium campylum* (*b*), the only ciliate which, in my experience, can cause confusion. *Glaucoma pyriformis* is distinguished by a conspicuous oral membrane, negligible gullet, and a round nucleus. In addition it grows vigorously in Difco yeast extract, while *Colpidium campylum* has never been grown in pure culture.[2]

Dr. André Lwoff very kindly compared my animal with his

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[1] A grant of money from the Brooks Fund defrayed a portion of the cost of this work.

[2] The "*Colpidium campylum*" reported upon by the author in 1932 and 1933, used at Stanford, and carried to Yale by Dr. Bond, is a typical *Glaucoma pyriformis*. A sub-culture of this strain was used by Elliott in his 1935 paper (personal communications from Dr. Jahn and Dr. Bond). Chatton never achieved the pure culture of *Colpidium campylum* (personal communication). The author has tried to grow this ciliate on the media described in the present paper without success—its nutritive requirements resemble those of *Colpidium colpoda*.

Glaucoma pyriformis, using his precise silver impregnation method. He found extremely slight differences in the silverlines. He also compared Dr. Butterfield's (1929) "*Colpidium*" with my *Glaucoma pyriformis*, finding no appreciable difference.

Dr. A. Kahl also advised me concerning the identification of this ciliate. He identifies it with his *Saprophilus oviformis* (Kahl, 1926, 1931), apparently synonymous with *Glaucoma pyriformis*. The species *Colpidium striatum*, inadequately described by Stokes (1886), he treats as a nom. nud.

Unfortunately the taxonomy of this species is not settled as yet, so the most well-known name is used here.

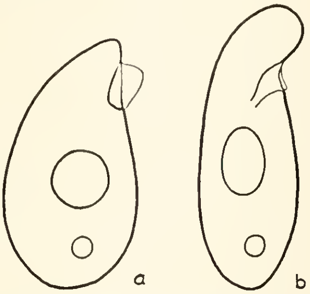

FIG. 1. *a*, *Glaucoma pyriformis* (Ehrb.) Schew.: 47μ. *b*, *Colpidium campylum* (Stokes) Bresslau: 55μ.

MATERIALS AND METHOD

General

The temperature for all experiments varied between 24.2° and 25.2° C.

Bacteriological methods for sterilization of apparatus and manipulation of organisms (flame technique) were used. A method for sterilizing protozoans has been reported (Hetherington, 1934*a*).

Glassware

Stock and experimental vessels were 125 cc. Pyrex Erlenmeyer flasks. Isolation vessels were Columbia dishes.[3] Graduated serological pipettes of 2-cc. capacity were used for stock transfer and for measuring inocula in the experiments.

Enrichment

Glaucoma pyriformis adjusts readily to 0.5 per cent yeast extract and proceeds to grow, while the other ciliates which have been obtained in pure culture are difficult to start in this medium.

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[3] Arthur H. Thomas Co., Philadelphia, Watch Glass No. 9851.

Transfer

Daily transfer of approximately 0.2 cc. was continued for one month or more, thus maintaining fairly constant and extremely vigorous growth, previous to quantitative experimentation.

It is essential to observe the following procedure in measuring the volume of inocula. Draw up the 2-cc. volume and allow to flow back into the flask at the same rate at which the volumes will be delivered, then (keep the culture stirred!) again draw up, slowly (i.e. at the speed at which it will be discharged), and deliver the desired volumes into the flasks. Do not use the final 0.4 cc. in the pipette.

Counting

A method of counting was described (Hetherington, 1934*b*). This has been refined to overcome a specific difficulty; a tendency on the part of *Glaucoma* to adhere to the surface of the pipette.

1. If dilution is required (if not, proceed directly with Step No. 2), draw up 10 cc. into a clean graduated pipette, then deliver 10 cc. back into the culture. Stir thoroughly. Again draw up 10 cc. and deliver into that volume of tap water in a beaker which will achieve the correct dilution.

2. Prepare 10 slides (or any number desired) by placing two separate drops of particle-free aceto-carmine on each.

3. Using a pipette (medicine dropper) whose drop-volume has been ascertained, draw up a volume larger than required for counting, and deliver drop by drop back into the culture. A convenient volume for each drop is 0.02 cc.

4. Stir thoroughly. Draw up a similar volume. Deliver one drop on top of each aceto-carmine drop, being careful to hold the pipette at the same angle at which it was calibrated. Do not deliver the final 2 or 3 drops.

The organisms were counted under the low power of a dissecting binocular. The number of organisms in the original culture can be calculated by obvious methods.

Accuracy of Counting Method

The following numbers are six successive counts, giving glaucomas per cc., from the same culture: 7,770; 7,070; 7,130; 7,710; 7,410; 7,610. This series shows the largest variation experienced. The mean is 7,450, with a probable error of 174.5, or 2.3 per cent. The majority of counts were more satisfactory than the above, giving a mean probable error of about 1 per cent.

The use of pipettes smaller than 2 cc. and with narrow delivery apertures decreases the accuracy because the ciliates adhere more irregularly.

Division Rate

The division rate, r, the number of divisions per organism per day, is given by the following formula:

$$r = \frac{\log Y - \log P}{t \cdot \log 2}$$

where $Y =$ total yield of ciliates, $P =$ inoculum, the number of ciliates inoculated, $t =$ time of growth in days.

If young organisms or those about to divide (i.e. considered as an inoculum of 2 for each dividing individual) are inoculated the following formula must be applied:

$$r = \frac{\log Y - \log (0.7071P)}{t \cdot \log 2}.$$

The factor 0.7071 expresses the approximate difference between the age of the individuals of the inoculum and the mean age of those of the yield.

MEDIUM

Inorganic

While this ciliate will, of course, grow on the complex nutrients, e.g. yeast extract dissolved in doubly distilled water, all experiments were carried out using a salt solution, Peters' medium, in place of the pure water. This medium, in addition to the very desirable buffering action, possesses at least the empiric virtue of resembling ordinary fresh waters, particularly with respect to its dilute nature and content of calcium bicarbonate.

Preparation

Put about ten grams of $Ca(OH)_2$ (Kahlbaum—*pro analysi*) into about a liter of doubly distilled water: cork and shake. Allow to stand for two days, shaking a few times in the interim. Pass CO_2 through this solution (at 23° C.), using either commercial tank CO_2 or a generator, for one hour. Cork and allow to stand for 24 hours. Pour off the slightly cloudy supernatant fluid into two good quality ground-glass-stoppered bottles. Pass CO_2 through each for a minute, stopper, and label: $Ca(HCO_3)_2$, 0.01 M., 55 cc./liter. Prepare 0.015 M. $MgSO_4$, K_2HPO_4, and 0.0075 M. Na_2HPO_4 in doubly distilled water, and add "10 cc./liter" to the label of each.

A liter of Peters' medium is prepared by diluting 55 cc. of the calcium bicarbonate and 10 cc. of each of the others to one liter. This medium should not be shaken into equilibrium with air before autoclaving.

Organic

The most striking characteristic of this particular strain of *Glaucoma pyriformis* is its complete failure to grow on peptone (or Neopeptone or Tryptone, Difco). Since it was found to grow luxuriantly on plain autoclaved wheat or on yeast (Fleischmann, pure dry), and since these media contain great numbers of particles, it was considered a "particle feeding" strain, and extensive measurements of growth were made using a medium of 0.5 per cent yeast, and 0.5 per cent dextrose.[4]

Since the advent of the new powdered yeast extract by Difco, it has been possible to show that particles are not the crucial requirement of my strain. Further, it has permitted a far closer approach to an ideal medium, particularly so when combined with certain powdered yeasts which were specially prepared under the direction of Mr. H. G. Dunham of the Difco Laboratories. Without his gracious coöperation in supplying these yeasts, the high division rates described in this paper could not have been achieved.

Clear 0.5 per cent Yeast Extract will support this *Glaucoma* with a division rate of 6.36. As stated, peptone will not support growth. What is the effect of adding a fine suspension of insoluble yeast particles to 0.5 per cent peptone? Difco extracted yeast no. B13412H was prepared for this purpose. When added in 0.005 to 0.05 per cent concentration, yielding cloudy suspensions, growth remained nil, just as in clear peptone. When raised to 0.1 per cent, however, considerable growth could be obtained. This is in contrast to growth in 0.5 per cent peptone + finely powdered whole yeast (No. B13412), where 0.005 to 0.05 per cent concentrations gave good growth. Raising the concentration to 0.1 per cent improved growth to a relatively small extent.

From these experiments it was concluded that minute quantities of the extractives of yeast complete peptone as a food for this strain of *Glaucoma pyriformis*, and that particles are of slight or no importance.

Peptone and yeast extract gave similar growth in the presence of 0.1 to 0.3 per cent whole yeast No. B13412. When the whole yeast was reduced to 0.05 per cent, the peptone medium gave weaker growth. The mean division rate on the 0.2 per cent and 0.1 per cent whole yeast + 0.5 per cent yeast extract was respectively 7.83 and 7.71. The following optimum medium was used for the measurements to be reported in this paper: 0.5 per cent yeast extract and 0.2 per cent powdered yeast No. B13412 in volumes of 50 cc. in 125-cc. Pyrex Erlenmeyer flasks. The surface/volume ratio is 0.55.

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[4] Note the similarity of this experience with that of Peters (1929). It seems to the author practically certain that Peters' "*Colpidium colpoda*" is *Glaucoma pyriformis*.

The finely powdered yeast is prepared in 1 per cent concentration which is autoclaved, 15 lbs. for 15 minutes. All particles must be wet before autoclaving, otherwise sterility may not be achieved. This is allowed to stand for 24 hours, then the cloudy supernatant fluid is poured off, separating from the solid residues. It is regulated to pH 7.0 to 7.2 with NaOH, and added to 1 per cent yeast extract in the correct proportion, then diluted: dispense into the flasks, cotton stopper, and autoclave.

Comparison of Different Media

Table I verifies quantitatively some of the relations just described, and permits comparison of a number of different nutrient media. Inoculum 15 is 1,490 glaucomas from a culture in 0.5 per cent yeast extract. Inoculum 7 is 717 from a culture in dextrose[5] 0.5 per cent, yeast No. B33425 0.25 per cent, and yeast No. B33413 0.25 per cent. The numbered yeasts are whole yeasts prepared in different ways. Cultures containing 0.5 per cent of whole yeast may for convenience be called whole yeast media, while those containing yeast extract may be called yeast extract media.

The most general relations observed are that inocula from whole yeast into whole yeast give the lowest division rates, from yeast extract into whole yeast the next highest, while the highest are those from yeast extract into yeast extract.

The chemically treated yeasts B33413 and B33425 contrast with B13412 (plain finely powdered whole yeast) in that the addition of dextrose improves the former and harms the latter (Experiments 3 to 15). Yeast B33425 also contrasts with B13412 in that fractional sterilization (85° C.) does not help, while marked improvement is noted for the latter (Experiments 8, 9, and 13, 14).

Finally, increasing the complexity of the medium beyond that of Experiment 18 results in no gain. The liver used in Experiment 21 is

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[5] Culture media containing dextrose turn strongly acid after inoculation of *Glaucoma pyriformis*, even in the presence of considerable phosphate buffer. The flasks containing dextrose (Experiments 2, 3, 4, 6, 7, 8, 11, 12, 13, 20, and 21) also contained excess $CaCO_3$, which maintained the hydrogen ion concentration within the limits of pH 7.30 to 6.83.

Table I

Growth of Glaucoma in different culture media. Fifty cc. of medium in 125-cc. flasks in all experiments. Inoculum 15 = 1,490 glaucomas, 7 = 717. Counts taken after 24 hours except for wheat which represents 72 hours of growth. *F. st.* indicates that the medium was fractionally sterilized.

Experiment	Medium	Inoculum	Yield	Mean division rate
1	5% Wheat	15	870,000	3.06 (3rd day)
2	0.5% Yeast (Fleischmann) 0.5% Dextrose	(many counts)		6.02
3	0.5% Yeast No. B33413 0.5% Dextrose	15	143,200	6.60
4	0.5% Yeast No. B33413 0.5% Dextrose	7	64,900	6.49
5	0.5% Yeast No. B33413	7	12,740	4.15
6	0.5% Yeast No. B33425 0.5% Dextrose	15	125,100	6.39
7	0.5% Yeast No. B33425 0.5% Dextrose	7	12,080	4.07
8	0.5% Yeast No. B33425 (*F. st.*) 0.5% Dextrose	7	13,300	4.21
9	0.5% Yeast No. B33425 (*F. st.*)	7	11,630	4.02
10	0.5% Yeast No. B33425	7	25,500	5.15
11	0.5% Yeast No. B13412 0.5% Dextrose	15	116,300	6.29
12	0.5% Yeast No. B13412 0.5% Dextrose	7	46,600	6.02
13	0.5% Yeast No. B13412 (*F. st.*) 0.5% Dextrose	7	71,000	6.63
14	0.5% Yeast No. B13412 (*F. st.*)	7	118,300	7.37
15	0.5% Yeast No. B13412	7	57,000	6.32
16	0.5% Yeast Extract 0.1% Yeast No. B33413	15	177,500 202,000	6.99

TABLE I (*Continued*)

Experiment	Medium	Inoculum	Yield	Mean division rate
17	0.5% Yeast Extract 0.1% Yeast No. B33425	15	213,700 211,500	7.16
18	0.5% Yeast Extract 0.1% Yeast No. B13412	7	68,500 69,200	6.59
19	0.2% Yeast Extract 0.2% Peptone 0.1% Yeast No. B13412 0.1% Kidney No. 23211	15	183,000 169,000 173,000	6.87
20	0.2% Yeast Extract 0.2% Peptone 0.1% Yeast No. B13412 0.1% Kidney No. 23211 0.4% Dextrose	15	152,000 149,000 155,300	6.70
21	0.2% Yeast Extract 0.2% Peptone 0.1% Yeast No. B13412 0.1% Kidney No. 23211 0.2% Dextrose 0.2% Eli Lilly Liver (unheated)	15	156,400 139,000 131,000	6.58

Eli Lilly Company's Extract No. 343, sterilized by passing through a Seitz bacteriological filter.

Hydrogen Ion Concentration

The reaction of the Difco yeast extract (prepared in Peters' medium) is pH 6.91–6.95, of the complete medium after autoclaving, 7.00–7.05, and after supporting growth of large numbers of *Glaucoma* for 24 hours, 7.00–6.97, as measured with a quinhydrone electrode.

Repeated efforts to duplicate Elliott's (1933) bimaximal growth in the regions of pH 6 and 7.5 with a low point at 7, failed. At pH values of 6.7 and 7.4 it was impossible to equal the yields of medium regulated nearer neutrality. Slow growth, finally yielding large numbers, was achieved at the following extremes: pH 4.35 and pH 9.37 ± .05.

Growth-promoting Characteristics of the Yeast

The apparent presence of an unstable growth-promoting substance in the fractionally sterilized yeast No. B13412 (Table I, Experiments 13 and 14) suggested some more tests, which are recorded in Table II.

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Experiments from inocula 844 and 1,696 were run simultaneously with those of Table III and are therefore strictly comparable. Even when the yeast is 0.1 per cent, half the concentration of the standard medium (p. 431), marked acceleration is evident.

The maximum rates of growth here recorded are not very high when compared with those of Enriques (1916). Working at a higher temperature and in the presence of bacteria he obtained as many as 13 generations in 24 hours, and never less than 9.

GROWTH FROM INOCULA OF 0.2 CC. CONTAINING VARYING NUMBERS OF GLAUCOMA

The volume 0.2 cc. was chosen simply because of its convenience. Table III gives unselected results of several experiments taken at different times and on different batches of medium. A comparison of the division rates resulting from inocula 581 to 1,696 indicates that average rate of growth is about constant over this range. When 4,270 are inoculated a distinct drop in rate of increase occurs, while 26,640 give only about 72 per cent as rapid growth. This is presumably a consequence of crowding.

TABLE II

Growth of *Glaucoma* on varying concentrations of fractionally sterilized (85° C.) whole yeast No. B13412. All media contain 0.5 per cent yeast extract. To be compared with corresponding inocula in Table III.

Whole yeast	Inoculum	Yield	Division rate
0.1%	844	242,000	8.16
0.1%	844	248,000	8.20
0.2%	1,696	617,000	8.50
0.2%	1,696	628,000	8.53
0.4%	1,330	549,600	8.69
0.4%	1,330	507,000	8.57

Inocula between 500 and 2,000 per 0.2 cc. are very readily obtained either from flasks which have been subject to daily sub-culture using a 0.2 cc. transfer volume, or from a two-day culture inoculated with one ciliate. Inocula below 581 were not tested because it is difficult to count the number of ciliates in such dilute cultures.

These results were unselected. There is every reason to believe that, with repetition of one kind of experiment using medium made up in large quantities, greater uniformity of yield could be achieved.

GROWTH FROM AN INOCULUM OF ONE DIVIDING GLAUCOMA

A dividing ciliate is a convenient inoculum because the age is known within a few minutes. It is here treated as an inoculum of two glaucomas. It was found that two days of growth gave numbers which could be accurately counted.

TABLE III

Relation between number of glaucomas inoculated and rate of growth. Volume of inoculum = 0.2 cc. Period of growth = 24 hours.

Inoculum glaucomas in 0.2 cc.	Yield glaucomas in 50 cc.	Division rate
581	146,000	7.97
	140,500	7.94
	138,500	7.90
	137,000	7.89
844	201,100	7.89
	204,900	7.92
	209,100	7.95
962	227,000	7.89
	228,000	7.89
	238,200	8.02
	241,800	8.02
1,696	417,700	7.95
	416,800	7.94
	407,800	7.91
	Mean	7.93
4,270	963,000	7.82
	968,000	7.83
26,640	1,483,000	5.80
	1,575,000	5.88
	1,560,000	5.87
	1,499,000	5.82

Table IV gives counts after 2 days and again after 3 days. Considering the 48-hour counts first, two differences are evident when compared with Table III, inocula 581 to 1,696. First, the mean division rate is lower, although continuity occurs at the upper extreme. Second, greater variability in division rates is evident. The 48-hour experiment was repeated with closely similar results, giving a mean division rate of 7.76. The mean division rate during the third day is 4.80. The uniformity of division rates for the 72-hour interval probably reflects a consequence of crowding.

Comparing inoculum 26,640 of Table III with the 48-hour yield of Table IV, it is seen that the latter is 2.5 times the former. The mean yields, on the other hand, differ by only 17 per cent (1,529,250-1,838,600). This suggests that these populations are practically static; i.e., they represent the "crop" for this particular strain of *Glaucoma pyriformis*. They are still very healthy organisms.

The Interval Required for Division

What is the actual behaviour of individuals in these flasks? Does each divide after a certain interval in a regular fashion, or are the cultures mixtures of degenerating and more or less rapidly multiplying ciliates? To answer qualitatively first, extensive observation of flask-cultured glaucomas under the microscope never revealed degenerating or conjugating forms; on the other hand, uniformity in appearance was a striking characteristic. When this strain was cultivated on Fleischmann's yeast (p. 430), a considerable proportion of the population always appeared to be conjugating. Closer inspection revealed two apposed longitudinal depressions which apparently developed some hours after fission. Such forms nourished and divided like the others. All traces of this phenomenon disappeared in two transfers on the Difco medium.

Table IV

Growth from an inoculum of one dividing glaucoma after 2 days and after 3 days.

48 hours		72 hours	
Yield	Division rate	Yield	Division rate
57,200	7.65	1,691,000	6.73
60,700	7.69	1,943,000	6.80
67,600	7.77	1,849,000	6.77
66,500	7.76	1,878,000	6.78
78,900	7.88	1,832,000	6.77
Mean	7.75	Mean	6.77
Mean for last day			4.80

The following experiments were carried out in Columbia isolation dishes enclosed in Petri dishes. One hundred or so glaucomas were removed from a young and thriving flask culture, put in a dish containing 1 cc. of medium, and watched through the Petri cover. When one was seen to be well along in division, it was removed with a pipette (medicine dropper) of generous bore to a similar dish containing

1 cc. of medium, and the time of separation noted. A series of such experiments was watched continuously at a temperature of 24.2° to 25.2° C. to determine the time required for the next fission.

A surprising result was obtained. Irregularity between the limits of 2 hours and 45 minutes for the shortest interval to 4 hours and 25 minutes for the longest, was observed. Daughter cells were as irregular as any others. The mean was roughly 3 hours and 25 minutes (3.42 hours). To give a mean division rate of 7.93 a division every 3.028 hours is necessary. The second division was also followed [6] but no significant difference in behaviour occurred.

EARLY GROWTH OF GLAUCOMA CULTURED IN FLASKS

If the marked irregularity and slowness of growth of organisms observed in shallow dishes is not due to incidental technical features of the experiment, it should be possible to demonstrate the same thing in flask cultures by counting before large numbers make their appearance.

TABLE V

Growth during the first 9-hour period compared with later growth. Inoculum = 1,696 glaucomas in 0.2 cc.

Growth during 9 hours		Growth during 24 hours	
Yield	Division rate	Yield	Division rate
11,470 10,710 9,965	7.35 7.11 6.82	417,700 416,800 407,800	7.94 7.94 7.91
Mean	7.09	Mean	7.93
Mean for last 15 hours			8.44

Table V gives results from an inoculum of 1,696 ciliates after only 9 hours, then again after 24 hours. The mean division rate for the first 9 hours is 7.09, lower than that for 24 hours, and gives a mean interval required for division of 3.385 hours. This agrees satisfactorily with the mean interval of 3.42 actually observed in the previous section. The mean interval required for division during the last 15 hours is 2.84 hours.

An objection could be raised to this experiment because the inoculum was from a culture containing large numbers of ciliates,

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[6] Ciliates other than the two under observation were removed; hence the second interval followed without manipulation by pipette.

8,480 per cc. (424,000), and might be "conditioned," so the shock of transfer to fresh medium would explain the early retardation.

Accordingly, in spite of the labor involved in counting small concentrations of ciliates, an inoculum of 0.5 cc. containing only 661 glaucomas was tried. Counts were taken after 6 hours, 12 hours, and 24 hours.

Table VI substantiates Table V in that a definite acceleration in rate of growth occurs during the 24-hour period. Since the six-hour rate (7.27) is higher than that for 9 hours (7.09), it is possible, as suggested above, that some shock of transfer plays a small rôle in the experiments of Table V. The mean rate of 8.55 for the last 12 hours gives an interval required for division of 2.8 hours: the glaucomas divide every 2 hours and 48 minutes.

Table VI

Growth during the first 6-hour period compared with later growth. Inoculum = 661 glaucomas in 0.5 cc.

Growth during 6 hours		Growth during 12 hours		Growth during 24 hours	
Yield	Division rate	Yield	Division rate	Yield	Division rate
2,497	7.67	8,540	7.38	166,000	7.97
2,331	7.27	8,660	7.42	168,500	8.00
2,163	6.84	8,760	7.45	168,000	7.99
Mean	7.27	Mean	7.41	Mean	7.98
Mean for second 6 hours			7.57		
Mean for last 12 hours					8.55
Mean for last 18 hours					8.23

The division rate for the first 6 hours gives an interval required for division of 3.30 hours. This value and that for 9 hours in Table V (3.385) agree satisfactorily with the mean, 3.42, of intervals actually observed in the preceding section.

The probable error of the 6-hour counts corresponds in terms of division rate to about 0.25, and of the 9-hour counts of Table V to about 0.2. The recorded order corresponds to the order in which the flasks were inoculated without refilling the pipette. Accordingly, the serial variability observed in the 6-hour counts and also in the 9-hour counts indicates actual difference in growth, and can be explained by assuming a loss of 30 to 50 ciliates on the side of the pipette at each inoculation.

The apparent lag in early growth recorded in Tables V and VI suggests the "allelocatalytic effect" of Robertson (Johnson, 1933, gives a good recent review of this subject). Definite conclusions concerning the nature of this lag in *Glaucoma* must await a technique which will permit accurate counting of small numbers.

Comparison with Other Strains of Glaucoma pyriformis

Ten different strains of *Glaucoma pyriformis* have been either isolated in this laboratory or sent here through the courtesy of other investigators. It is possible to distinguish between a few of them morphologically, and between every one of them by simple counts of growth in the yeast medium.

In spite of the fact that this medium was developed specifically for my yeast-requiring strain, three other strains, including Dr. Elliott's "*Colpidium striatum*" (1933), which resembles Lwoff's strain in that it grows luxuriantly on peptone, grow better on it. The latter divides about 1.05 times as fast as mine. The most difficult strain to distinguish from mine is Dr. Butterfield's (1929) "*Colpidium,*" which is interesting in that it will grow for a few transfers in peptone, then fails to increase. It divides about 1.03 times as fast as mine. This form is thus intermediate in its nutritive requirements between the more typical peptone-growing strain and the yeast-requiring variant described in this paper. The other strains divide less rapidly, the lowest division rate recorded being 6.23, about 0.84 times as fast.

Morphologically, a slender ("schlanke form") strain is the most striking variant: it is distinctive even when very well nourished. The author is acquainted with this strain through the courtesy of Dr. Elliott and Dr. Kahl.

Glaser and Coria (1935) obtained from hot springs strains of *Saprophilus oviformis* (= *Glaucoma pyriformis*) which grow vigorously at 37° C.

Summary

1. The strain of *Glaucoma pyriformis* reported upon in this paper will not grow in peptone in pure culture, or in this plus a fine suspension of insoluble particles of extracted yeast.

Yeast extract (Difco), particle-free, supports a division rate of 6.86.

Whole yeast (Difco—autoclaved) plus either peptone or yeast extract supports division rates from 7.65 to 8.02.

Media more complex than this do not increase the division rate.

If the whole yeast added to the yeast extract is fractionally sterilized rather than autoclaved, marked increase in division rate results.

2. There is but one optimum hydrogen ion concentration. This is in the region of pH 7.

3. When 600 to 2,000 glaucomas are inoculated in 50 cc. of medium, the division rate is 7.89 to 8.02 for the first day.

When larger numbers are inoculated the division rate is lower.

4. If one dividing glaucoma is inoculated, the mean division rate (48 hours) varies from 7.65 to 7.88.

5. Observation of isolated organisms in shallow dishes revealed marked irregularity in the interval required for division: from 2 hours and 45 minutes for the shortest interval to 4 hours and 25 minutes for the longest. The mean was 3 hours and 25 minutes, corresponding to a division rate of 7.

6. Counts of growth in less than 24 hours apparently demonstrate a lag in early growth. No definite conclusions were drawn because of the large error resulting from counting low concentrations of ciliates.

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REGENERATION IN THE POLYCHÆTE CLYMENELLA TORQUATA

III. Effect of Level of Cut on Type of New Structures in Anterior Regeneration

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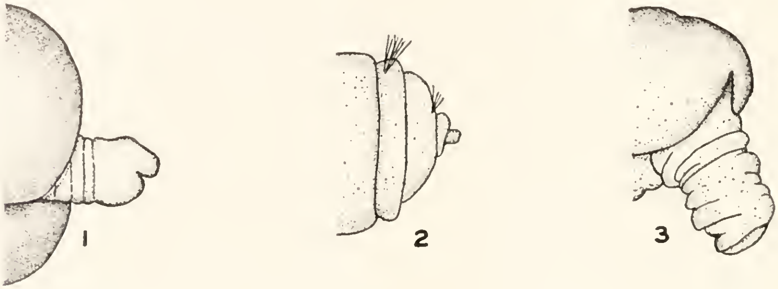
Although many polychæte annelids regenerate posteriorly, relatively few readily form new heads. The succession of events in anterior regeneration in *Clymenella torquata* after the loss of 5 segments has already been described (Sayles, 1932). At that level regeneration is usually complete after about fifteen days. During the summers of 1929–31 a record was kept of regenerates among worms as they were brought into the laboratory. Of 1,364 specimens of *Clymenella*, 33 were regenerating anteriorly, none of these at levels posterior to the sixth segment. Under laboratory conditions it has been possible to secure a number of regenerates at the tenth segment and some even at the eleventh.

In this paper anterior regenerates at various levels are discussed. These were obtained during work carried out at Woods Hole from 1928 to 1934, inclusive. The materials and methods have already been discussed in a paper on posterior regeneration in this worm (Sayles, 1934).

Experimental Results

Anterior regeneration after the removal of from *1 to 6 segments* may be considered first. Upon cutting at these six levels the mortality of posterior pieces was relatively low, 85 per cent or more of the pieces surviving for 10 days or longer. After the loss of one or two segments a complete bud was formed by the tenth day. This was also usually the case after the loss of three segments. At the fourth and fifth segments the majority of buds were complete after ten days but about one-third of the pieces required 11 to 15 days to form a bud which showed complete external segmentation, parapodia and folds associated with the peristomium. At the sixth most of the pieces were just beginning to show external evidences of segmentation after 10 days and, in most cases, they were complete, as far as general external features were concerned, by the fifteenth day.

Of the specimens cut at the *seventh segment* about 70 per cent survived. Here, as at the sixth, organization of the new bud was slower than at more anterior levels. In addition one piece failed to produce a complete bud of the usual shape. Instead there was formed a peristomium of ordinary size and structure but followed by five, possibly six, segments of much smaller diameter. This specimen was observed early in this work (in 1928) and unfortunately it was neither carefully drawn nor preserved for further study. This bud was probably of the type which has appeared on regenerates at the eighth segment and somewhat more posterior levels. All other pieces at this level which survived for 15 days had produced complete new buds by that time.



Three regenerates at the eighth segment. Drawn with aid of camera lucida.

FIG. 1. Ventro-lateral view of weakly segmented bud after 12 days. × 16.

FIG. 2. Right-lateral view of regenerate, at 21 days, which had probably broken off at a very slender region in the middle of a bud. × 13.

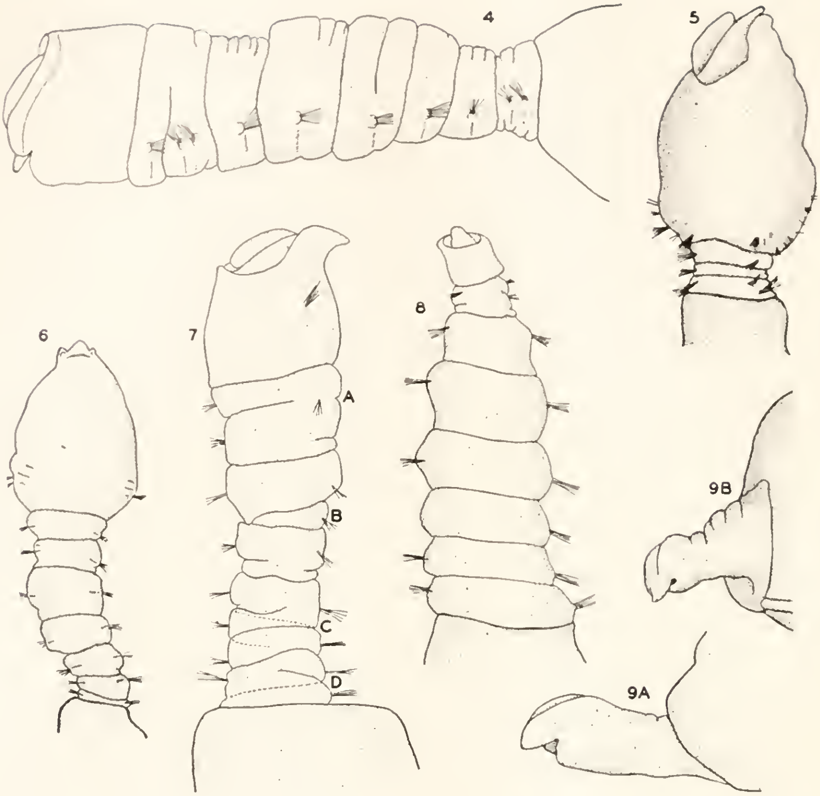
FIG. 3. Right-lateral view of a somewhat crude bud after 18 days. × 16.

At the level of the *eighth segment* about 30 per cent of the pieces cut were dead after 10 days. Of the 24 survivors 21 had small buds which were either non-segmented or only weakly segmented (Fig. 1). Two others had buds consisting of one or two distinct segments of good size, anterior to which was a small unsegmented region (Fig. 2). In these two pieces it might seem that the proximal segments have become established without the development of an organized peristomial region. A study of anterior regenerates at more posterior levels, however, suggests that in each of these two cases a bud may have been formed with a very slender mid-portion. Buds of this latter type have been observed at the ninth, tenth and eleventh segments. Some of these have been broken although considerable care was taken to keep them intact. The portion of the bud then remaining attached to the old part was similar in appearance to the arrangement of the new tissue observed in these two specimens at the eighth segment. In the case of the remaining piece, regeneration progressed very slowly. The bulb of gut, formed in wound closure, did not disappear until the third day and no new tissue was apparent until the seventh. On the tenth day only a very small bud had been formed. After 18 days this worm had produced a complete, well-formed bud. Three of the others had died. Of the latter two were the ones which had probably been broken previous to the tenth day. At this time 15 had formed complete buds consisting of a head region followed by seven setigerous segments of medium diameter. Another piece had a bud consisting of a well-developed head region followed by a slender part of indistinct segmentation. This worm died on the twenty-third day without showing any marked change in the meantime. Two others had heads of the type shown in Fig. 3. The other two were of the broken type described above, but the result of recent breaks.

At the eighth segment, therefore, the majority of survivors replaced accurately the segments lost. Some, however, produced very slender buds but none of these pieces survived long enough to complete segmentation. A few produced buds which were indistinctly segmented and had but small head folds.

At the *ninth segment* about 70 per cent of the pieces survived ten days. After twenty days 37 out of 77 were alive. These 37 may be placed in 8 groups according to the type of bud formed. (1) Nine had formed complete buds of 8 setigerous segments plus an asetigerous head region, all of these of the usual shape and size. (2) Four had produced buds having the full number of segments, but with the third to eighth segments, inclusive, of a diameter much smaller than that of the first two. (3) Each of 4 others had a well-developed head region followed by a slender part. The anterior portion of the latter showed about four setigerous segments and the posterior part, near the original cut piece, was indistinctly segmented. (4) Seven had short buds of medium diameter with head regions of the normal type. One consisted of 6 segments and one of 4 segments. Each of the other 5 had a peristomium and 3 or 4 setigerous segments followed by a weakly segmented region with no parapodia visible. (5) One consisted of 10 segments the last 3 of which were a little irregular. (6) Three had short conical buds without any constrictions apparent. (7) One had not regenerated beyond wound closure. (8) Eight had buds of various shapes with peculiar arrangements of both parapodia and segmental constrictions.

Several of the last-named group merit more detailed descriptions. One of them (Fig. 4) had a bud which was divided externally into nine



Seven regenerates at ninth segment. Drawn with aid of camera lucida.

FIG. 4. Bud of good shape except for extra pairs of parapodia on third and ninth segments. Twenty-nine days. Lateral view. × 10.

FIG. 5. A bud with proximal portion segmented and distal part swollen and unsegmented. The number of setæ in each group on the swollen region is accurately shown. Twenty-eight days. Dorsal view. × 10.

FIG. 6. Another bud with distal part swollen and unsegmented. Twenty-nine days. Ventral view. × 6.

FIG. 7. Bud possessing split-segment (*A*), intercalated segment (*B*), left-hand spiral of one and one-half segments (*C*), and right-hand spiral of a little more than one-half segment (*D*). Ventral positions of intersegmental grooves, where they do not match the dorsal ones, are shown in dotted lines. Twenty-eight days. Dorsal view, somewhat lateral in anterior part. × 10.

FIG. 8. Another bud showing irregularities confined mostly to distal part of bud. There was no left parapodium on what was probably the third segment. The notopodium (but not the neuropodium) of the seventh segment was missing. Twenty-nine days. Dorsal view. × 10.

FIG. 9. Two somewhat weakly developed buds. *A*. On the tenth day. Left lateral view. × 16. *B*. On the twelfth day. Left ventro-lateral view. × 12.

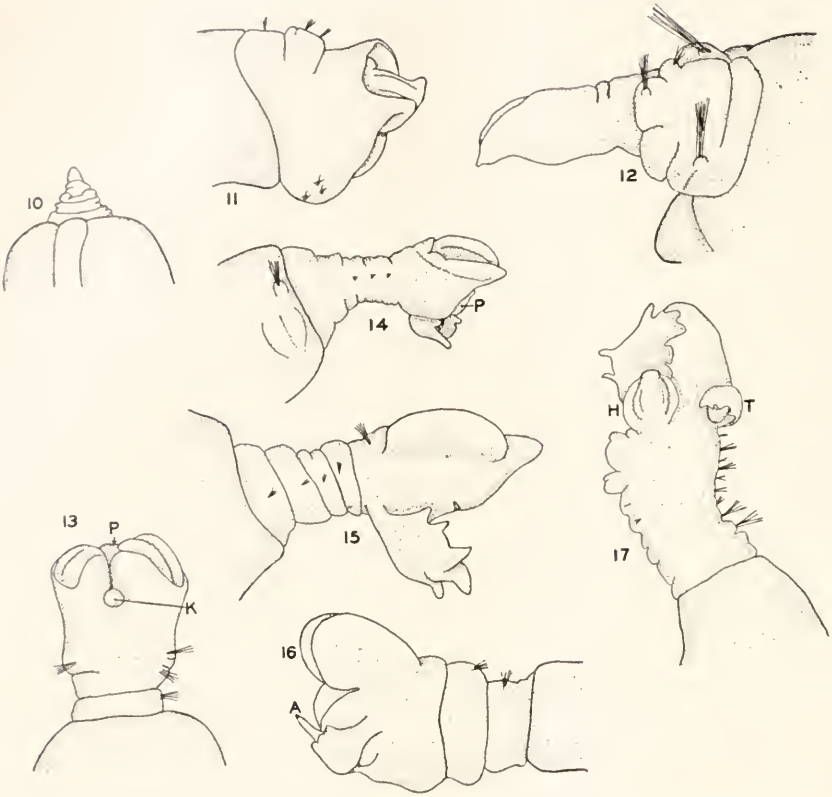
segments, the first of which was asetigerous and of ordinary shape. A peculiar thing about this bud was that the third and ninth segments each had two pairs of parapodia. Both neuropodial and notopodial portions were present. Aside from this point the bud was normal in appearance. If, on microscopic examination, it is found that each of these double regions belongs really to 2 segments, it is clear that the piece has formed 11 segments after the loss of 9. If not, then there has at least been a duplication of parts. It is also interesting to note that these unusual regions were some distance from one another, one next to the old part and the other near the distal end of the bud. Another of these pieces (Fig. 5) consisted of three proximal segments, apparently of the usual type, in front of which was a somewhat swollen unsegmented region, the anterior part of which bore considerable resemblance to the usual type of first segment. The posterior part of this region bore a number of clusters of setæ, none of which were of the neuropodial type. Three of these clusters were of the same appearance as ordinary notopodia but the others consisted of only 2–4 setæ each. Here, then, there is a case of completion of the three proximal segments, and, for the most part, of the peristomial segment. In between, however, was a region where external segmentation and parapodium-formation had proceeded in an unusual manner. Another of these peculiar regenerates had formed a bud consisting of a somewhat swollen anterior part followed by a slender region composed of seven segments, each provided with neuropodia and notopodia of the ordinary type (Fig. 6). The portion of larger diameter was unsegmented. At its anterior tip it had weakly-developed peristomial folds. Near its posterior end it bore several groups of setæ. On the right side there were two notopodia, ventral to which were three vertical rows of uncini, the central row being longer and bearing uncini larger than either of the others. On the left side there was but one notopodium, but here also there were three vertical rows of uncini all of about the same size. Here, then, we have a case where organization of the posterior two-thirds of the bud had gone on in the usual manner but that of the anterior part had been abnormal.

A fourth individual (Fig. 7) in this same group consisted of a peristomium, bearing a pair of parapodia, and the apparent equivalent of 8 additional setigerous segments. The latter, however, showed several peculiarities of arrangement. Immediately behind the peristomium was a region consisting of a single segment on the right but split on the left to form two parts. Next there was a regular segment. Then a separate half segment was present on the right side. Behind this was another regular segment which was followed by a spiral segment beginning on the dorsal side and running to the left one and one-half turns to end on the ventral side. Behind this was another spiral region running in the opposite direction and apparently ending laterally on the left side. It was very difficult to determine the exact limit of this adjacent to the old part of the worm. Although this last region might possibly be interpreted as an intercalated half-segment, it has more the appearance of a very short spiral. The fifth and last one of this group to be described is shown in Fig. 8. The posterior two-thirds of the newly formed bud was of the usual diameter. The remaining portion was of about one-half this size. The larger posterior portion of the bud consisted of five setigerous segments with only one point that was unusual, namely, the absence of the left notopodium from one segment although the neuropodium was present. Anterior to these five segments was another complete setigerous segment, of intermediate size. The smaller anterior part of the bud consisted of two main divisions. The anterior one was clearly a peristomial region. The prostomium was, in proportion, shorter and thicker than usual. The head folds on the peristomium were lower and broader than those ordinarily found in this region. The posterior division of this smaller region was incompletely marked off into three parts, the anterior of which bore a parapodium on each side. The middle one of these small parts bore a parapodium on the right side only; the posterior one was without parapodia. In this case, also, the region near the old part was organized in the usual manner and the anterior part of the bud, farther away from the old piece, underwent an unusual organization.

Posterior pieces regenerating anteriorly at the *tenth segment* were much more susceptible to infection than pieces cut at more anterior levels. Of 195 pieces cut only 83 (or about 43 per cent) survived for ten days. At this time, 35 had formed buds of about the type shown in Fig. 9. Thirty-six had produced buds which were either very short or had irregular shapes. Ten showed no external evidence of new tissue. One was slightly irregular with some resemblance to a small heteromorphic tail. The other survivor had formed a bud, the main part of which did not differ greatly from Fig. 9. From the right side of this main part there projected a somewhat lobulate structure arising at a level slightly posterior to that of the mouth. Unfortunately, this specimen died on the fourteenth day before there had been time for many noticeable changes from this condition. At this time, it was similar in appearance to the early stages in the formation of multiple structures such as those described below.

At the end of 20 days there were 55 survivors, which might be placed in 5 groups. (1) Two had small buds in the early stages of head formation and segmentation. Both of these died at about this time without undergoing any marked changes. (2) Five showed no external evidence of new tissue. It should be noted, however, that one of these had a slender bud at 10 days. Apparently this bud was lost and not replaced, possibly because of insufficient time. These were in the same condition when they were discarded or died after 20–24 days. (3) Four had produced irregular buds. One of the latter, a short weakly segmented bud, was drawn (Fig. 10) and killed at this time. Another (Fig. 11) had a short bud somewhat bloated in appearance and deep red in color. It had fairly well formed head folds and prostomium, but showed practically no evidence of segmentation. On each side were 3 notopodia, rather irregularly arranged especially on the right side. The third of these buds was short and conical. Nine days later this bud (Fig. 12) consisted of a short, broad proximal portion with two pairs of parapodia and a distal part similar in appearance to a typical head bud of about ten to twelve days. This may possibly have been a re-regenerate. The last of these buds was a peristomium followed by a short unsegmented region. On this bud were several extra knob-like growths which looked like the beginnings of multiple structures. Unfortunately this animal died at this time and before any drawings of it had been made.

(4) Six others of the survivors at 20 days had multiple structures. One consisted of a single, weakly segmented bud with a mouth which was practically terminal. The dorsal side of the peristomium was double with two complete parts, each consisting of head folds enclosing a prostomium. This double appearance was first unquestionable on the twentieth day. By the following morning the animal had died. No drawing is available. Another bud (Fig. 13) consisted of 3 setigerous segments and a double peristomial region with its parts so turned that the sides ordinarily ventral were more or less facing one another. Between the latter there was a single mouth with a small proboscis exhibiting the usual activity. Three of the other buds were similar in that each was a well-developed bud of several segments with a peristomium of quite good shape and with the second part a heteromorphic tail (Figs. 14, 15 and 16). There can be no question that these extra parts were tail buds. In the first place, the cirri, so characteristic of the anal collar of a normal worm, were present. Also these extra buds were almost identical in appearance with some of the unusual tails produced in posterior regeneration at the eighth to tenth segments. In different posterior regenerates at these levels many intermediate forms between these odd types and the usual one have



Eight regenerates at the tenth segment. Drawn with aid of camera lucida.

FIG. 10. Ventral view of a weakly developed bud. The segmentation was very indistinct on dorsal side. No parapodia were visible. Twenty days. × 12.

FIG. 11. Right dorso-lateral view of short, swollen irregularly segmented bud of about 4 segments. Twenty-five days. × 12.

FIG. 12. Left view of a bud irregular in portion near old part. Twenty-nine days. × 24.

FIG. 13. Bud with anterior part of peristomium double. *P*, proboscis. *K*, knob of tissue. Twenty days. × 22.

FIG. 14. Right view of regenerate with ventral tail outgrowth. *P*, proboscis. Twenty days. × 10.

FIG. 15. Right view of regenerate with ventral tail outgrowth. Twenty-six days. × 16.

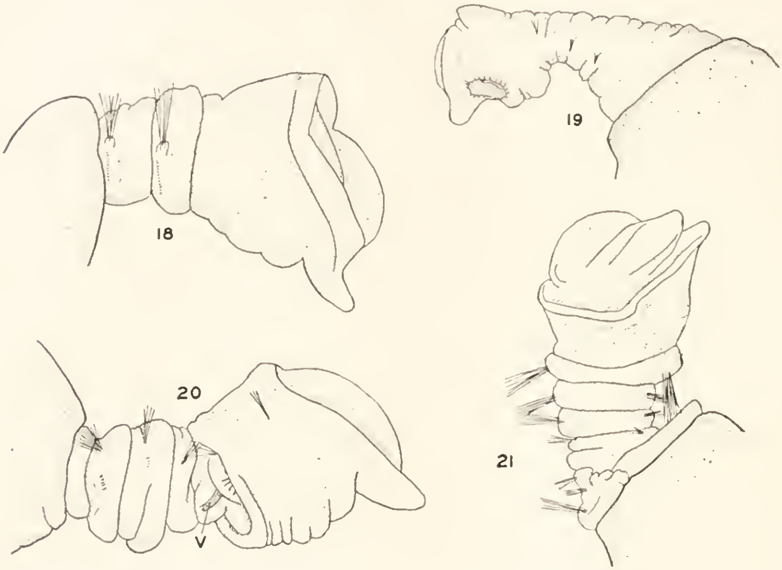
FIG. 16. Left view of regenerate with tail outgrowth on left side. *A*, anal cone. Twenty-six days. × 16.

FIG. 17. Dorsal view of heteromorphic tail with small extra head (*H*) and tail (*T*) outgrowths Twenty days. × 16.

been found (Sayles, 1934). In two cases the heteromorphic tails arose from the mid-ventral region somewhat behind the mouth. In the third instance the small tail-bud arose mid-laterally at approximately the same longitudinal level as the mouth. This point of origin matches the position of the circumœsophageal connective. The last of these buds was the one which, on the tenth day, resembled a heteromorphic tail. By the thirteenth day this bud clearly consisted of several rather irregular segments terminating in an incomplete anal collar, open on the left side and bearing 6 cirri. At this time there were present in the vicinity of the base of the collar two rather large outgrowths without any apparent differentiation into head or tail type. These two extra parts gradually assumed distinct shapes until after 20 days the condition shown in Fig. 17 existed. It may be noted that at this time there were at least 9 new segments anterior to which was the multiple region. The main terminal part was an anal segment bearing a half-collar on which were 7 cirri. From the right dorsolateral region at the base of this terminal segment arose a small bud including a partial anal collar with about five cirri and also a long anal cone. In the vicinity of the dorsal end of the open anal collar and somewhat to the left of the small extra anal segment there was the other small bud which showed the main distinguishing characteristics of a head segment. An interesting point is that this segment was in the proper position with relation to the old part despite the fact that it did not appear until after the main heteromorphic tail was clearly organized.

(5) The remaining 38 individuals alive at this time all had peristomial regions of ordinary size and shape. There was, however, considerable variation in the setigerous regions. For instance, one bud had two post-peristomial segments with no evidence of setæ. Two others had two such segments but with parapodia (Fig. 18). Three had a peristomium bearing a pair of parapodia at the level of the posterior end of the peristomial crown; 2 of these had 5 other setigerous segments and one had 4. Ten others had regular setigerous segments varying in number between 5 and 9. Two others had a number of segments but only 3 (Fig. 19) and 4, respectively, with parapodia. Each of these 18 buds was of fairly uniform diameter throughout its entire length. One of the other 20 consisted of the peristomium followed by 6 setigerous segments. The right side of the second setigerous segment was swollen and bore 2 extra parapodia. The remaining 19 in this group had slender proximal regions, 17 of them with regularly formed setigerous segments varying in number from 3 to 8. One of the others (Fig. 20) consisted of 6 or 7 segments

of which 4 had parapodia. In addition there was a pair of clusters of notopodial setæ far forward on what appeared to be a part of the peristomium. The proximal part of the bud was very irregular. The last of this group (Fig. 21) had a well-formed peristomium followed by 4 setigerous segments. Posterior to these there was an asetigerous region. Between the latter and the old part, on the left side only, there was an irregular structure bearing 3 notopodia. As a result the bud as a whole was tipped to the right.



Four regenerates at the tenth segment. Drawn with aid of camera lucida.

FIG. 18. Three-segment bud of good shape. Twenty-eight days. × 16.

FIG. 19. Left view of a bud at 27 days. × 12.

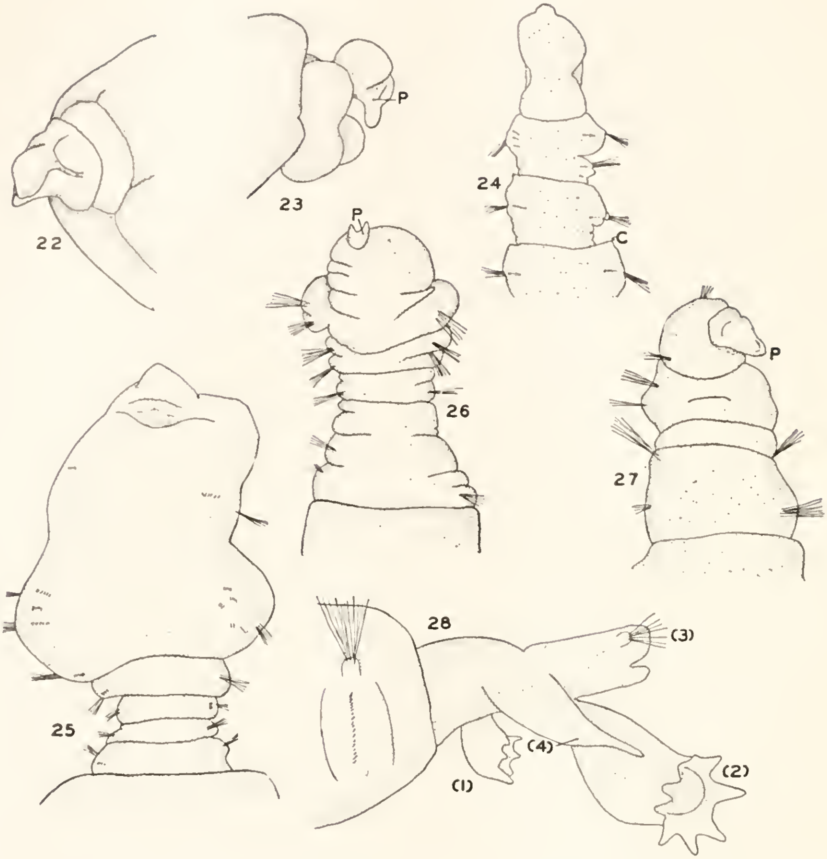
FIG. 20. Right ventro-lateral view of a somewhat irregular bud. V, ventral blood vessel. Twenty-three days. × 16.

FIG. 21. Dorsal view of regenerate with region bearing 3 notopodia intercalated on left at base of bud. Twenty-eight days. × 16.

At the *eleventh segment*, of 113 pieces cut, 30 (about 26 per cent) survived for 10 days. Of these, 11 showed no external evidence of new tissue. Four had short, unsegmented buds with but small head-folds. One had a double structure consisting of a heteromorphic tail from the ventral side of which another, smaller tail had grown out. One had a short heteromorphic tail terminating in an anal collar which was open ventrally and possessed 5 or 6 anal cirri. The other 13 had irregular buds of various types. Two of these were killed and 14 others died during the next 10 days. One of those killed (Fig. 22) had a bud composed of a somewhat irregular peristomium followed by a single asetigerous segment.

Of the 14 survivors at 20 days, 2 were without new tissue. Neither of these changed in appearance up to the times of death, one at 27 days, the other at 30. On the thirteenth day the single heteromorphic tail had dropped off. Little if any further new tissue was formed by this piece up to the time of its death on the thirtieth day. Four others had buds with peristomial regions of fairly definite shape followed by slender regions of 4–7 setigerous segments of quite regular arrangement. One worm had a bud (Fig. 23) made up of several irregular parts, the most anterior of which was apparently a much misshapen peristomial region.

Five other buds were 3–6 mm. long but irregular. Four of these survived until the twenty-eighth to the thirty-second day at which time they were drawn and killed. One, the anterior part of which is shown in Fig. 24, had a peristomial region followed by 8 setigerous segments, 2 less than were removed. The peristomium was of fair shape but with no trace of a mouth. It was followed by a region including at least two segments (which we shall call the second and third). These were separated from one another on the left but not on the right. It may be noted that there was an irregular arrangement of parapodial parts here. The fourth and fifth segments were of usual appearance, including the collar characteristic of the fifth segment of this species. Clearly then the 2 missing segments of this bud must have been omitted from its posterior part. The second of these buds (Fig. 25) included a posterior part of 4 setigerous segments anterior to which there was a swollen region a little over 2 mm. long and terminating in a peristomium of very nearly the usual appearance. This swollen region bore a number of groups of setæ of irregular arrangement. The third of these buds (Fig. 26) varied in diameter in different regions. The anterior part of the bud was without setæ but was constricted in several places. At its anterior tip was a very small knob of tissue, notched anteriorly. Aside from this knob, which might possibly be considered as a dwarfed, irregular peristomium, none of the characteristic head-features was present. The remainder of the bud was somewhat indefinitely marked off into segments. Judging on the basis of notopodia and pronounced constrictions, 7 or 8 segments were present in this part. The last of these buds consisted of 8 setigerous segments gradually reducing in size from the old part toward the tip. The anterior half of this bud is shown in Fig. 27. Anterior to the setigerous segments was a small region twisted somewhat dorsally. The latter looked like a minute, early peristomium.



Seven regenerates at the eleventh segment. All except Fig. 28 drawn with aid of camera lucida.

FIG. 22. Two-segment bud with somewhat irregular peristomium. Fifteen days. × 16.

FIG. 23. Right view of irregular bud probably including a peristomium (*P*). Nineteen days. × 16.

FIG. 24. Ventral view of anterior part of a bud which lacked a mouth. *C*, collar on fifth segment. Twenty-eight days. × 16.

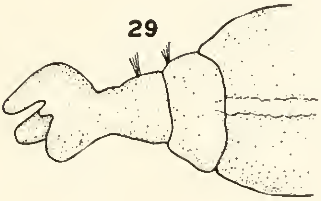
FIG. 25. Ventral view of a bud, the anterior part of which was swollen and unsegmented but possessing a number of groups of uncini as well as several notopodia. Twenty-eight days. × 16.

FIG. 26. Dorsal view of a regenerate of 28 days. *P*, possibly a dwarfed, irregular peristomium. × 16.

FIG. 27. Dorsal view of anterior part of bud which included three more segments. First two divisions twisted so that peristomium (*P*) is hooked dorsally. Thirty-six days. × 22.

FIG. 28. Right lateral view of a multiple bud at 22 days. The original main heteromorphic tail (2) was tipped somewhat ventrally by the development of two outgrowths—(3) and (4)—on dorsal side of bud. An extra, ventral tail (1) was also present. × 17.

The other survivor at this time was the one previously noted as having two tails. By the twenty-second day not only had these become distinct tails but also two other outgrowths from the bud had appeared (Fig. 28). Consequently, at this time there were present on the regenerating part the following structures: (1) A tail growing out from the mid-ventral line near the base of the new part. This bore a distinct collar region open on one side and bearing 6 cirri—a condition frequently found in tail-regeneration at mid-body levels. (2) A tail which had been terminal but which was now tipped ventrally. This tail had an anal collar and 9 cirri. The dorsal portion of the collar and the 4 cirri found there were shorter than these parts in the ventral region. This bud bore two pairs of notopodia near its base. (3) and (4) On the dorsal side of the mid-part of the terminal tail a newly-formed outgrowth was present as a result of which the tail was tipped ventrally. This outgrowth consisted of two parts—a larger one lying in line with the longitudinal axis of the worm and terminating in a bilobed region with two notopodia both on the more dorsal lobe; a slender, slightly longer part extending to the right (of the original posterior piece of the worm) and ventrally. The dorsal blood vessel could be seen running forward and dividing to send a branch into each of two dorsal parts. In spite of all precautions this worm died during the following night.



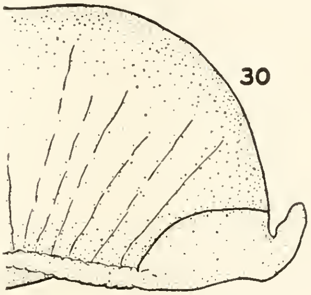


FIG. 29. Ventral view of a bud at twelfth segment after 17 days. It was probably a heteromorphic tail. × 13.

FIG. 30. Right lateral view to show bud formed at fourteenth segment after 33 days. Drawn with aid of camera lucida. × 22.

Regenerates at *levels posterior to the eleventh segment* may be considered together. No data on the relative numbers of deaths and survivals at these levels are available. Unfortunately a number of experiments involving these were started without making any record of the original numbers of pieces. At the end of 10 days there were 115 survivals at these levels and in the experiments where records of cutting were kept 92 had died. The actual total number of deaths was probably 8 to 10 times that number. Of the survivors, 59 still had bulbs of gut present and no new tissue evident and 55 had closed wounds but were without new tissue. One, regenerating at the twelfth segment, had a very short bud.

Twenty days after cutting there were 66 alive. Twenty-four of these had bulbs; 40 had the wound closed but no new tissue; 2 had small buds. One of the buds—at the twelfth segment—(Fig. 29) was probably a somewhat peculiar heteromorphic tail with 2 setigerous segments. This worm died on the twentieth day. The other regenerating piece (Fig. 30) had been cut at the fourteenth segment. After 33 days the new tissue present included a fairly large round mass over the wound surface from the anterior ventral part of which a slender portion extended forward and curved toward the dorsal side.

Discussion

In anterior, as in posterior, regeneration *Clymenella* tended to replace the segments lost. When six or less segments were removed they were exactly replaced. When 12 or more segments were removed, with 2 exceptions, no new bud was formed. In the intermediate region the percentages of survivors producing complete buds of approximately the usual shape were: at the seventh segment, practically 100; at the eighth, 75–85; at the ninth, 35–40; at the tenth, none out of 55 pieces; at the eleventh, none out of 14. At the last three levels many incomplete or irregular buds were formed. This is almost the exact reciprocal of the situation in posterior regeneration. In the latter no regeneration occurred in the anterior region, complete regeneration usually occurred in the posterior region, and in the intermediate region there was a progressive drop in the ability to regenerate as the cuts were made at more and more anterior levels.

It is interesting to compare regeneration in the laboratory with that under natural conditions. Of 1,364 worms examined during the summers of 1929–31, inclusive, only 33 showed evidence of having regenerated anteriorly. Of these, 12 had regenerated at the second segment, 10 at the third, 7 at the fourth, 2 at the fifth and 2 at the sixth. All of these had replaced exactly the parts lost. No worms were found which had replaced more than 6 segments. Since autotomy occurs only in the posterior half of the body, these regenerates must have resulted from accidental injuries. As such injuries could occur equally well in the middle and posterior regions of the body, it seems that under natural conditions such injured pieces are rarely, if ever, able to survive and to regenerate.

As in posterior regeneration, there was a fairly close parallel between the level of the cut and the ability to survive. In the region of the complete replacement of lost parts—at segments 1–6, inclusive—more than 85 per cent survived for 10 days or longer. In the region where buds were practically never formed—segments 12–21, inclusive—probably less than 15 per cent survived on the average, with only an occasional piece living after cuts at the most posterior levels. In the intermediate region there were the following percentages of survivals at 10 days: at segments 7, 8 and 9 about 70 each; at segment 10, 43; at segment 11, 26.

The response of *Clymenella* to the loss of an anterior portion of the body is different from that of most polychætes. We may mention a few cases, especially among genera having representatives in the Woods Hole region. According to Michel (1898) *Phyllodoce, Nephthys, Eulalia* and *Capitella* will not regenerate anteriorly. We have verified this for the first two. Czerski and Nusbaum (1905) kept capitellids for 25–27 days without the appearance of new buds. Czwiklitzer (1905) found that *Ophryotrocha* would produce new parts only when no more than portions of the first segment were lost. On the other hand, Berrill (1928) reported that *Chætopterus* would replace lost anterior segments up to and including the fourteenth. Posterior to that level anterior regeneration did not occur. This was an extension of the facts reported earlier by Gravier (1909). Pflugfelder (1929) found that *Diopatra* would rapidly replace anterior segments lost, apparently up to as many as fourteen.

In between these extremes are the cases where anterior regeneration occurs but only a few of the lost segments are replaced by newly-formed ones. In many cases the bodies of these worms are differentiated into regions, usually including a head, a thoracic region of a definite number of segments and an abdominal one of a variable number of segments. Vaney and Conte (1899) reported that *Spirographis* formed 2 new segments anteriorly after cuts in the anterior part of the body. The requisite number of thoracic segments was then obtained by metamorphosis of the abdominals. Watson (1906) found that *Potamilla* formed a head and one thoracic segment and then completed the thorax by metamorphosis. Berrill (1931) reported that *Sabella* produced a new head plus one or two collar segments and then completed the process by a metamorphosis of the necessary number of abdominal segments.

Clymenella does not fall exactly in any one of these three categories. It does tend toward a complete replacement of the lost part at anterior levels. In the mid-body region, in addition to many cases of irregular buds, there were a number in which the usual arrangement of things occurred but not all of the lost segments were replaced. Perhaps one of the best examples of this is the worm which produced a peristomium and only 2 setigerous segments, all of good shape, after the loss of 10 (Fig. 18). This worm was kept 28 days yet it showed no evidence of a change in the nature of the body-wall in which there is ordinarily a very definite line of demarcation (at the ninth–tenth intersegmental region) between the distinctly thicker wall anteriorly and the very thin wall in the posterior part. As might be expected from this fact, no trace of a new fifth-segment collar appeared on the old twelfth segment. Also in none of these cases where buds deficient only in the number of segments were formed was there any indication of the addition of new segments at the posterior end of the body. Since this worm has a constant number of segments—22—there is no growing region at the posterior end. It might then be thought that there was not the right kind of cellular material for such additions, although such material does appear when a piece of the worm is amputated. The suggestion came to mind that, if a few segments were removed from the posterior end of a regenerate, deficient as the result of the formation of such an anterior bud, then the new posterior bud might contain more segments than the part cut off. Vigorous looking worms with anterior buds of this special type were not common. After these cuts for posterior regeneration were planned, only two regenerates developed which could be used for this purpose. One of these—after 21 days regeneration at the tenth segment—had produced 3 new segments to form a bud similar to the one mentioned above. The other piece had regenerated 4 new segments at the ninth after 26 days. Both were cut again at the eighteenth segment. The former died a little less than 2 days later and without the completion of wound closure. After 15 days the other produced a new bud consisting of 4 distinct segments. Since the full number of segments to be present in a bud in *Clymenella* is laid down at approximately one time, it does not seem probable that more would have been added to this bud if it had been kept longer. This evidence is, of course, too scanty for us to draw any conclusions from it. For instance, the result might possibly have been different if the second cut had been made 12–14 days or less after the worm was brought into the laboratory.

As in the case of posterior regeneration in *Clymenella*, it seems that these buds of good shape, but consisting of a small number of segments should be attributed, in part at least, to a scarcity of material. It should be borne in mind that the mid-region of the body is a rather specialized one. For example, it is in segments 7 to 10, inclusive, that the large nephridia of these worms are located.

In several cases, 2 of which are shown in Figs. 7 and 20, a pair of parapodia was present on the peristomium. In most species of polychætes, including *Clymenella torquata*, parapodia are not present on the first segment. This occurrence in certain buds might be interpreted as reversion toward what has been considered the primitive condition.

In anterior regeneration 11 cases of multiple or heteromorphic structures have been observed. In contrast with this, neither of these features was observed in posterior regeneration in *Clymenella* (Sayles, 1934). Two of these were duplications in the peristomium. Two others were head-buds of the early type with extra outgrowths similar to the early stages in the formation of multiple structures. Both of these died before any more definite developments. Two others were heteromorphic tails without extra structures. Three were weakly developed heads, each with a secondary tail-bud outgrowth. One was a heteromorphic tail bearing 2 extra parts, one a head, the other a tail. The remaining one of these was a heteromorphic tail with an extra tail and also two other rather large, extra parts which had no characteristic head or tail features. If we consider these structures from the point of view of the levels at which they developed, we find that 8 were at the tenth segment, 2 at the eleventh segment, and 1 at the twelfth. To understand the true frequency of these structures we may note that 7 of the 8 at the tenth segment were alive at 20 days; this represents 14 per cent of the 50 survivors which had produced new tissue. At this time the 2 at the eleventh segment were 17 per cent of the 12 new buds at this level. The one at the twelfth was the only bud produced at that level by any piece. In other words, this type of irregularity occurred with about the same frequency at the tenth and eleventh segments and in the only case of a bud differentiating posterior to the eleventh. In contrast to the situation in posterior regeneration in this worm, there was here a definite upset in the organization of the new material with the result that the new structure was frequently not of the same type as the one lost.

One further point of interest was the appearance of a bud showing spiral segmentation, a split segment and an intercalated half-segment. This is the only case of this type among several thousand regenerates of this worm which have been studied. Also no intact worm has been found which possessed such arrangements.

Ehlers (according to Buchanan, 1893, p. 541) suggested that intercalated segments were the result of regeneration of lost parts. Buchanan implies that Ehlers thought the irregularities to arise at the point of contact of old and new. Speaking for herself, she says, "Even if the intercalation of half-segments were shown to be due to regeneration, it would still remain to be shown that the spiral arrangement is likewise due to it. The fact of spirals occurring in *Pentastomum*, where, as far as we know, regeneration does not take place, seems to me against this hypothesis." Morgan (1895), on the other hand, is of the opinion (p. 429) that "The large number of abnormal forms found in *Amphinome* is in part due no doubt to the frequent regeneration of portions of the body that takes place. The segments are broad, and for that reason it is surprising to find abnormal combination of the metameres so frequent." Unquestionably these features are very rare in *Clymenella*, in which the segments are extremely long. Also there can be no doubt about the fact that these peculiarities arose in regeneration. As far as the question of their appearance at the point of contact of old and new is concerned, the two spirals were adjacent to the old part. On the other hand, the half-segment and the split segment were at the distal part of the bud and separated from the spiral regions by a segment of apparently normal arrangement.

In connection with the origin of these peculiarities both Cori (1892) and Morgan (1892) thought that they were due to irregularities in the sizes and shapes of the developing mesodermal blocks. They attribute a spiral to a mesodermal block of one side, but not the other, being larger above than it is below, or vice versa. As a result, the single block of one side joins two of the other side. Then in another place this is offset to end the spiral. This bud in *Clymenella* is in agreement with such an explanation since there are the same number of segments on the right and left sides of the body, despite the fact that 4 irregular parts exist in a relatively short region.

SUMMARY

1. Six or fewer anterior segments were exactly replaced.
2. Only 2 worms produced buds at cuts posterior to the eleventh segment. Most pieces cut at such levels died relatively soon.
3. In the region from the seventh to the eleventh segments there was an increase in deaths, a decrease in ability to produce complete buds, and a marked increase in the number of irregular buds.
4. At the ninth to eleventh segments a number of buds were of the usual type but possessed fewer segments than had been removed. Available evidence points to the conclusion that regenerates of this type do not undergo any metamorphosis of segments or any increase in number of segments at the posterior end of the body.
5. In several buds a pair of parapodia appeared on the peristomium which is ordinarily asetigerous.

6. Eleven cases of heteromorphic or double structures appeared, all confined to cuts at segments 10, 11 or 12. Two of these were double parts of the peristomium. Two were heads with extra structures possessing neither head nor tail characters. Two were heteromorphic tails without extra structures. Three were heads, each with a secondary tail outgrowth. Two were tails with extra outgrowths.

7. A most interesting bud appeared on one piece cut at the ninth segment. It possessed the following features: a segment split on the left side, a half-segment intercalated on the right side, and two spiral segments. One of the latter was a left-hand spiral of one and one-half turns, the other a right-hand spiral of possibly a little over one-half turn.

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APICAL DOMINANCE AND POLARITY IN MNEMIOPSIS LEIDYI, AGASSIZ[1]

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The locomotion of *Mnemiopsis leidyi*, as it moves along with its oral end forward near the surface of the water, serves to demonstrate that this animal possesses a definite polarity. The forward movement is a continuous reaction and is interrupted only when the animal bumps into some definite obstruction. Even this interruption is very brief, for immediately afterward the animal resumes its oral direction of movement. This locomotion of *Mnemiopsis* demonstrates a definite polarity within the rows of plates since the beating of the plates causes the animal to move, and this in turn indicates the presence of some sort of dominance. The proof of this dominance, with its location and character, has been reported by Child (1917, 1933) and Coonfield (1934). The conclusion reached thus far is to the effect that the apical organ, or region, exerts a dominance over the other parts of the body. In support of this conclusion, I offer further experimental evidence which is listed and discussed according to the following five headings: (1) Swimming-plate movement, (2) feeding reactions, (3) reversing parts of plate-rows by grafting, (4) reversing the mid-body section by grafting, (5) transplanting the apical organ.

SWIMMING-PLATE MOVEMENT

The rows of swimming-plates of *Mnemiopsis* may be grouped into quadrants according to both their morphological and physiological features. Each quadrant contains two rows of plates, an adesophageal and an adtentactular (Figs. 3 and 4). These two rows may be considered as a unit which is coördinated with each of the other three similar units. This coördination is clearly shown by the synchronous movement of the plates as the animal progresses through the water. All plates stop beating instantly when the animal bumps into an object. Immediately after their cessation, either the swimming-plates reverse the direction of their effective beating for a very brief time, or the oral lobes contract. By either reaction, the animal is driven backwards for a short distance. In either effect, it seems clear that

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[1] Contribution No. 13.

we can assume that some sort of centralized structure or region exerts a dominance and regulation over the locomotor organs. The view that this dominance is located in the apical end of the animal is supported by the origin and sequence of the regular movement of the plates. The plate at the apical end of each row contracts first, then is followed by each other plate in the row contracting in sequence. This successive beating produces a metachronal wave, according to Parker (1905). The direction of this wave is never reversed, even on an isolated part of the animal in which a reversal of the effective direction was induced (Coonfield, 1934).

FEEDING REACTIONS

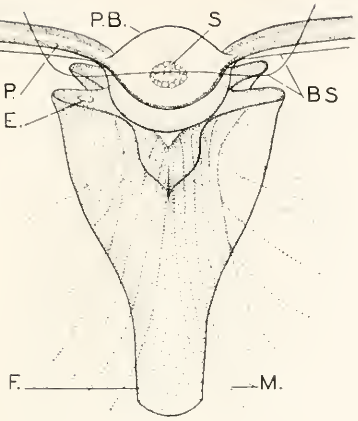
The feeding movements of *Mnemiopsis* were followed easily by observing specimens as they fed on clam juice containing a suspension of carmine. Every individual fed readily on this juice and the carmine particles made the tracing of the food passage, both on the outside of the body and within the body, quite convenient.

The clam juice was placed successively in very small quantities on all parts of the body surface, the entire group of reactions in response to each small quantity of food being recorded before more clam juice was placed on any other part. Positive feeding reactions followed only when the juice was placed on the adtentacular region, nearer the mouth than the apical end, on the lips, auricles, and lobes near the mouth (Fig. 3). The adtentacular region was chosen as the area on which to observe the feeding reactions principally because of its mechanical advantages. Therefore, the feeding reactions recorded here are those observed on the adtentacular region of the body.

As soon as the clam juice touched the adtentacular body surface, the swimming-plates of both rows nearby stopped their beating. This was due possibly to the intervention of the primary inhibitory action as described by Göthlin (1920). The very brief period of cessation was followed immediately by a reversal of the effective beating, which was also of very brief duration. Immediately after this reversal, the swimming-plates resumed their normal direction of beating toward the apical end of the animal. This reversal, though very brief in duration, was sufficient to initiate the moving of the food toward the mouth. While being moved in this direction, it spread out over the oval surface of the adtentacular region of the body (Figs. 3 and 4). In this region, the food was formed into a thin sheet by the addition of mucus from the body wall of the animal and was carried on toward the mouth and into the labial trough (Fig. 4) by the beating of very small cilia which cover this part of the body. Thence it was propelled on to the mouth both by cilia and by the peristaltic contractions of the labial ridge (Fig. 4). The tentacles lying at the edge of the labial trough were caught in the food stream and were stretched out in the direction of the mouth. Some of them were even broken off, presumably by the current, and were taken into the mouth with the food. The above description of the feeding reactions of *Mnemiopsis* follows fairly closely that of Main (1928). He makes no mention, however, of the reversal of the swimming-plates, nor does he report the presence of cilia on the body surface of the animal. The lips were primarily responsible for pushing the food into the mouth. The food then passed on up the stomodeum (stomach) to the funnel (Fig. 3) by the effective beating of cilia which cover the entire lining of this region, and by the peristaltic contractions of the wall of the stomach. At the funnel, the food, in solution and in suspension, was propelled out into the paragastric, the tentacular, and the meridional canals by the beating of cilia which cover all the inner surfaces of these canals (Figs. 3, 4, and 5). The association of a meridional canal with the swimming-plates is shown in Fig. 5. Some food in the funnel was carried also up into the infundibulum (Fig. 1) by cilia, where it either returned to the lower funnel region and was carried out into the other canals or was discharged. Within one or two hours after an animal fed, it discharged a steady stream of materials through a pore in one angle of the infundibulum (Fig. 1). This pore, with its discharging reaction, has been reported by Main (1928). I am not certain, however, whether the pore is permanent or only temporary. All the animals that I observed discharging materials ejected them through only this one particular region. The pore is visible only when the actual discharge is taking place.

REVERSING PARTS OF PLATE-ROWS BY GRAFTING

The metachronal wave in each row of swimming-plates originates at the apical end and moves toward the oral end of *Mnemiopsis*, while the effective beat of these plates is toward the apical end. The origin and progression of the metachronal wave supports the view that the apical zone is responsible for the initiation of this wave. This zone, therefore, might well be considered as a region of dominance. Further support of this view is indicated by the synchronous rhythm exhibited by all plates of the body in their metachronism. That the rows of plates are polarized and are regions through which impulses pass, resulting in the movement of plates, has been reported by Child (1933), Coonfield (1934), and Parker (1905).



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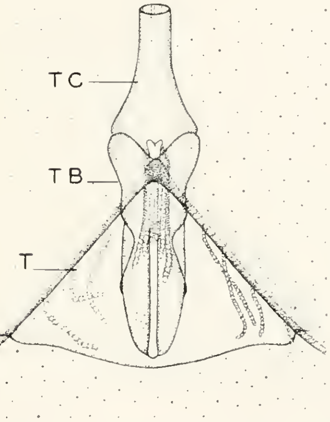

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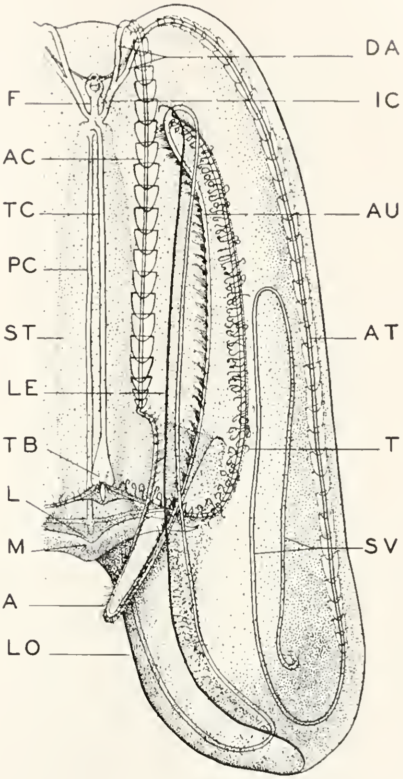

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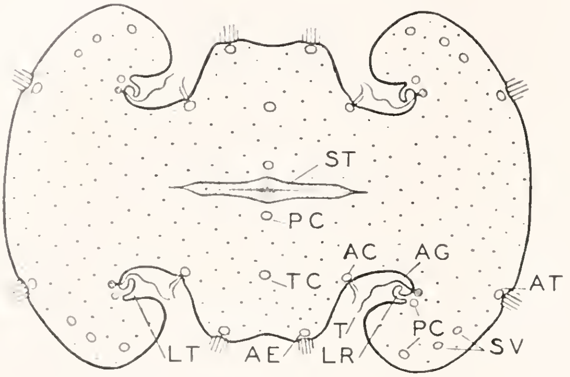
FIG. 1. A drawing of the infundibular funnel and polar body of *Mnemiopsis*. *BS*, body surface; *E*, excretory pore; *F*, infundibular funnel; *M*, muscles which terminate in the body wall; *P*, polar plate; *PB*, polar body; *S*, sense organ.

FIG. 2. A drawing of a tentacular bulb of *Mnemiopsis*. *T*, a lateral tentacle; *TB*, tentacular bulb; *TC*, tentacular canal.

FIG. 3. Drawing of a lateral view of *Mnemiopsis*. *A*, auricle; *AC*, adtentacular meridional canal with swimming-plates; *AT*, adesophageal meridional canal with swimming-plates; *AU*, auricular canal with plates; *DA*, adradial canals; *IC*, interradial canal; *F*, infundibular funnel with the polar body and sense organ above it; *L*, lip; *LE*, edge of lobe; *LO*, lobe; *M*, mouth; *PC*, paragastric canal; *ST*, stomodeum; *SV*, subventral meridional canal; *T*, lateral tentacle; *TB*, tentacular bulb; *TC*, tentacular canal.

Since the impulses which pass along the rows of plates are polarized, while these rows are in their natural position and orientation, the proof of the permanency of this polarity, which would test also the view of apical dominance, is revealed to some extent in the following experiments. A wedge-shaped piece of the mid-piece of either a single row or a similar section containing two adjacent rows, was cut out, rotated 180°, and grafted back in the same location. The adtentacular rows were cut and grafted because of certain mechanical advantages.



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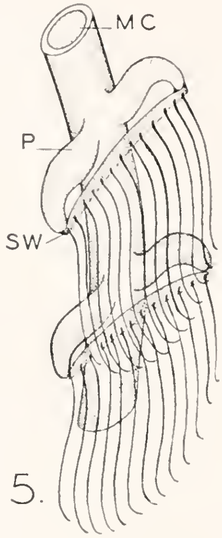

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Fig. 4. A diagrammatic cross-section of *Mnemiopsis* in the region of the oral ends of the adtentacular rows. *AC*, auricular canal and plate; *AE*, adtentacular meridional canal and plate; *AG*, auricular groove; *AT*, adesophageal meridional canal and plate; *LR*, labial ridge; *LT*, labial trough; *PC*, paragastric canal; *ST*, stomodeum; *SV*, subventral meridional canals; *T*, lateral tentacle; *TC*, tentacular canal.

Fig. 5. A diagram of a meridional canal to show its association with the swimming-plates. *MC*, meridional canal; *P*, branch of the canal to the base of a plate; *SW*, swimming-plate.

The ends of the rows of the grafts were placed in contact with the ends of the intact rows. In this new location, the rows of the grafts were exactly opposite, morphologically and according to their polarity, to these characteristics of the intact rows. The grafts were held in place by strands of cotton for about two hours to permit sufficient healing. During the healing period, the movement of the plates within the grafts was not in harmony with the synchronism of the other plates of the body, though these were perfectly synchronized.

The plates, however, in a single graft were synchronized with each other and the direction of both their metachronal and effective beating was the same as before rotation. In grafts involving a single row of plates, this condition existed until the effects of healing and regeneration gave the following result. Within thirty hours after the grafts were made, a canal, regenerating from the end of each of the intact

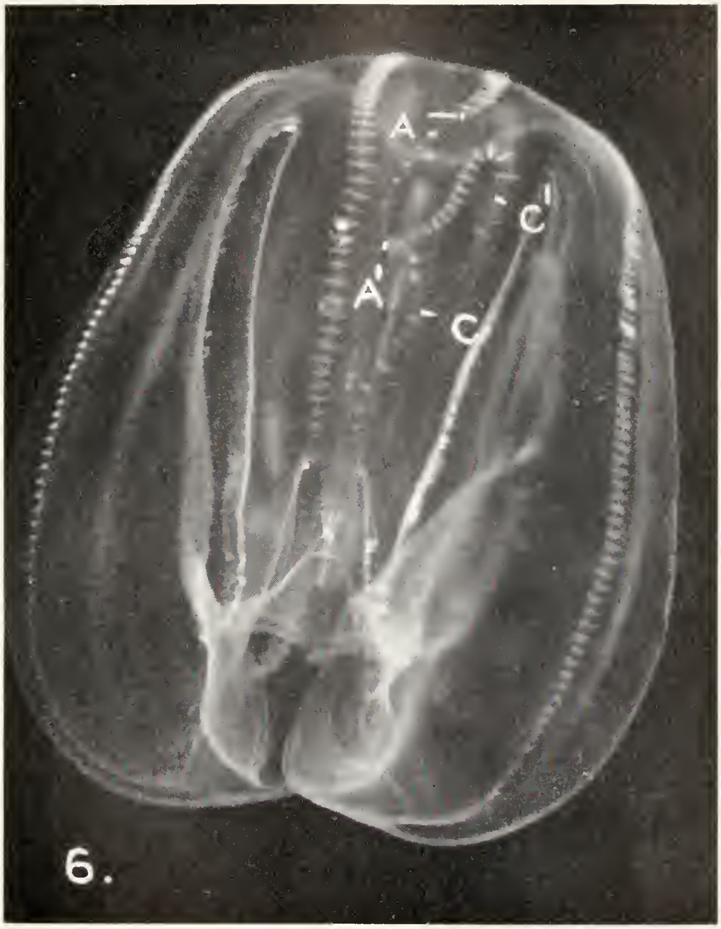

FIG. 6. This is a photograph of a living animal showing the result of rotating a piece of an adtentacular row. *A*, the point where the row was cut near the apical region; A^1, the end of the graft which was joined to the intact row at *A*. The row between *A* and A^1 shows the regenerated row. *C*, the point where the row was cut near the oral region; C^1, the end of the graft which was joined to the intact row at *C*. The row between *C* and C^1 shows the regenerated row. The row between A^1 and C^1 represents the rotated graft.

oral part and apical part, made connection with the corresponding morphological end of the graft (Fig. 6). At this time, the beating of the plates of the graft became synchronized with that of the other plates. The rotated grafts containing sections of two adjacent rows of plates responded in the following manner. The tips of the rows at each end of the graft moved in toward each other. The ends of the intact rows at the graft region also moved toward each other. The rows of plates remained in this state, neither fusing with each other nor regenerating, as in the case of the experiments involving only one row. Here again the metachronism and the effective beat of the plates in the graft continued as had been established before the rotation was made. The total actions exhibited by these grafts in the matter of independence of plate movement were the same as those of a single, isolated piece, as reported by Coonfield (1934), who stated that the plates of an isolated piece beat as a coördinated unit.

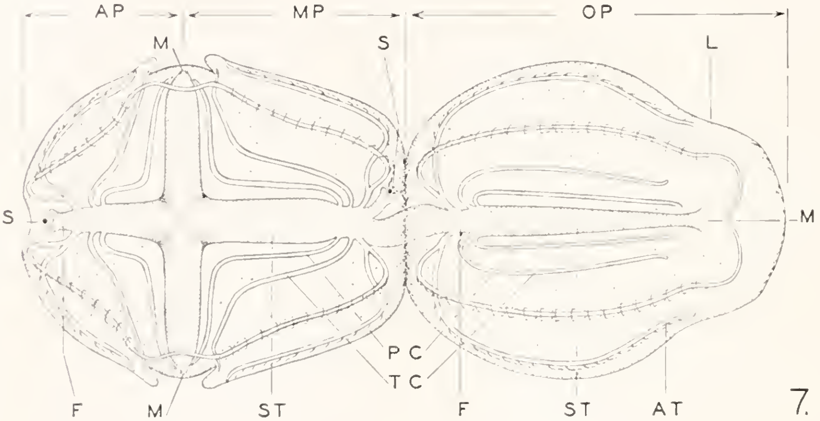

FIG. 7. A drawing of a living animal which had its mid-section rotated and grafted back in place. *AP*, apical piece; *AT*, adtentacular canal and plates; *F*, infundibular funnel; *L*, lobe; *M*, mouth; *MP*, mid-piece; *OP*, oral piece; *PC*, paragastric canal; *S*, sense organ; *ST*, stomodeum; *TC*, tentacular canal.

REVERSING THE MID-BODY SECTION BY GRAFTING

In order to test further the problem of apical dominance and the established polarity of impulses within the rows of swimming-plates, as set forth in the preceding section of this report, the following experiments were executed. The body of *Mnemiopsis* was cut across twice between the apical and oral ends, thus dividing the body into three parts, an apical, a middle, and an oral one (Fig. 7). The mid-section was rotated 180° and then grafted in place between the apical and oral sections. During healing and fusion, the three pieces were held together by strands of cotton and strips of filter paper. The mid-piece contained only the parts of the rows of plates and the digestive tract that are naturally in this region (Fig. 3). The apical piece contained the apical organ, the funnel and infundibulum with the interradial canals, and the bases of the meridional canals under the rows of plates. The oral piece contained the mouth, the tentacular bulbs (Fig. 2), the lobes, the auricles, and the ends of the meridional canals in the plate rows. Due to the severity of the operation and the mechanical handicaps accompanying it, only a few of the experimental animals lived. In most cases the oral piece of these operatees failed to fuse, leaving only the apical and the mid-piece healed together. Both the two-piece grafts and the three-piece grafts were retained and observed.

The results obtained from those with the three parts healed together are shown in Fig. 7. The mid-piece regenerated an apical organ and a funnel with its canal connections. The stomodeum of the middle piece fused with that of the apical piece. At the region of the fusion, digestive tubes, with their mouths and lips, were regenerated. The corresponding meridional canals of the two pieces joined. The oral piece failed to regenerate an apical organ, but an infundibulum with its canal connections was formed. This structure fused with the infundibulum of the mid-piece (Fig. 7). Food, taken in at the mouth of the oral piece, passed up its stomodeum and into the same structure of the mid-piece. Also, in these experimental animals, the swimming-plates of each piece behaved as though the piece constituted an individual organism. Thus the metachronal and the effective beating directions in the plates of the mid-piece in its rotated position were opposite to those of the apical and oral pieces. In all experiments wherein only the apical and the middle pieces joined, the mid-piece regenerated an apical organ and an infundibulum with its canal connections. The corresponding meridional canals and stomodeum of each failed to fuse. These operatees subsequently either separated or died.

TRANSPLANTING THE APICAL ORGAN

An apical organ (Fig. 1) of one animal was grafted to the body of another animal, about midway between its apical and oral ends and on the adtentacular surface. This transplant contained some of the funnel and the bases of the interradial canals. The grafts healed in very quickly, and within two days the regenerating ends of their interradial canals joined the adradial canals of the host. These new canals developed new plates which, according to the directions of their metachronal and effective waves, were coördinated by the transplant. Some of these plates, due to their relationship to those of the host and to their regulation by the transplant, beat in a direction opposite to that of the respective rows of the host. Within ten days after the transplant had healed in the host, it had been completely absorbed. This absorption was a continuous one, lasting about four days, during which time the transplant became less vigorous on each succeeding day.

Discussion

Any region of an organism which exerts a regulating influence over body activities, such as growth, regeneration, and the transmission of impulses or substances to other parts of the body, is regarded as possessing physiological dominance. This physiological dominance, according to Child (1928), may possess a correlative factor which undergoes a decrement in intensity with an increase of distance from the point of origin. Then a region of *Mnemiopsis* if it exerts a regulating influence over the other parts of the body possesses a physiological dominance. Such a region, the apical one, is located in a depression at the aboral end of the animal. It contains a polar body (apical organ) which lies just above the tip of the infundibulum. This polar body (Fig. 1) is made up of the following visible parts. 1. A concentration of lithic materials in the shape of a morula enclosed within a bell-shaped capsule. This capsule is composed of fused cilia (Mayer, 1912) and is supported upon four ciliated grooves which connect each with an interradial canal. 2. A polar plate which lies on the surface of the depression, surrounds the capsule, and extends to either side of the capsule in the stomodeal plane. 3. A concentration of nervous elements which radiate out into the plate rows. These elements are thought to be responsible for the coördination of the plate movements.

The swimming-plates of the eight rows of *Mnemiopsis* are synchronized as a unit in their beating while the animal is in its natural condition. This synchronism of the eight rows is rather significant, since, according to Coonfield (1934), each row shows the highest type of unity in the animal. That these units are regularly brought under the control of a particular region, presumably the apical organ, is convincing in demonstrating that this organ is a region of dominance.

The feeding reactions show the presence of a secondary polarity which is subjective to the primary one. This secondary reaction is temporarily dominant during feeding, requiring the presence of food as a stimulus. That this secondary polarity is not permanent is shown by the temporary reversal of the swimming-plates in the region stimulated. This reversal is rarely executed except during feeding. Just after the food is taken into the mouth, the primary polarity becomes dominant again and supercedes the secondary one.

The permanency of the polarity of the rows of plates, which I believe supports the view of apical dominance, is shown very definitely by the experiments in which the mid-section of a row was rotated and grafted back in place. The polarity of the rotated section of row had been so well established previous to the operation that it was maintained throughout the healing and regenerating period. This is significant since in its new location this polarity was just opposite the primary polarity of the body. The permanency of this polarity was further demonstrated by the directed regeneration of the ends of the apical and the oral parts of the row. Here the end of the apical part was in contact with the oral end of the graft, but instead of fusing with this physiologically opposite end, the apical part regenerated a canal which avoided the graft until it reached the end with which it had been originally in contact. The end of the oral part was in contact with the apical end of the graft, but instead of fusing with it, the oral part regenerated a canal which also avoided the graft until it reached the end with which it had been in contact. The experiments involving parts of two adjacent rows did not show any regeneration. It is probable that the size of the rotated piece mechanically prevented regeneration of rows. Despite the fact that regeneration did not take place, the ends of the rows of the rotated piece did not fuse with those of the apical or oral parts.

The experiments involving the rotation of the mid-section of the body of *Mnemiopsis* confirm the interpretations of the experiments cited in the preceding paragraph and show further that the apical organ exerts a dominance over the other parts of this animal. This additional evidence is brought out by the regeneration of an apical organ at the aboral end of the rotated piece. Apical polarity had been so firmly established in the body of the animal before the operation that the apical end of the graft formed an organ even though it was joined to the part of the body having an apical organ. I believe that during the period between the cutting of the animal and the actual fusion of the middle and the apical pieces the correlative factor had established a dominance in the apical end of the mid-piece. This dominance promoted the regeneration of an apical organ. Further indication of this correlative factor lies in the fact that the oral piece failed to form this organ. The oral piece fused with the mid-piece and its regenerated infundibulum joined that of the mid-piece.

The fact that the transplant in the experiments involving the transplantation of an apical organ behaved at first as an independent unit is significant. During the first few days after the operation, it maintained this unity. However, after four days, this unity of action was less marked. This decrease in independence was carried to completion within ten days, at which time absorption was complete.

This view of apical dominance is supported by Child (1917), who found that in response to KCN treatment the cessation of rhythmic plate-movement of *Mnemiopsis* occurred first at the aboral end of a row. He reports further that, according to the relation between susceptibility and general metabolic condition, the aboral end of a row is the region of highest metabolic rate in this gradient, and from this point, the rate decreases in the oral direction. Hanström (1928), in discussing the subepithelial nervous tissues of cœlenterates, believes that the diffuse plexus is transformed into a longitudinal axis, and in the ctenophores, this axis is connected with the apical organ. The opinions of these two investigators are confirmed by the experiments cited and discussed in this report.

Conclusions

1. Apical dominance in *Mnemiopsis* is indicated by each of the following: *a.* The plates of all eight rows are synchronous in their beating. *b.* Following temporary reversal of the effective wave during feeding, the original direction of this movement becomes reëstablished. *c.* Apical polarity within a single row persists even though a mid-section of the row is rotated by grafting. *d.* A rotated mid-piece retains its original polarity and regenerates an apical organ. *e.* A transplanted apical organ retains its identity at first, but eventually becomes absorbed.

2. The intensity of dominance in the normal animal decreases as the distance from the apical region increases.

3. Apical dominance and its resultant effects account for the various activities and environmental responses of the organism.

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DECOMPOSITION OF ORGANIC MATTER IN SEA WATER BY BACTERIA

III. FACTORS INFLUENCING THE RATE OF DECOMPOSITION[1]

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The question has been raised by Krogh (1934*a*, *b*) and other investigators, whether or not the organic matter in sea water can be utilized by bacteria and can be broken down with the liberation of the constituent elements in mineralized forms. It has been previously reported (Waksman and Carey, 1935*a* and *b*) that the organic matter in sea water is not in a resistant state, but can be readily attacked by the bacteria found in natural water, when this water is placed under favorable conditions. These investigations have further brought out the fact that this organic matter is sufficient to support an active bacterial population. The rapidity of decomposition of the organic matter by bacteria is most conveniently determined by the rate of oxygen consumption in the water and by the liberation of the nitrogen in an available form; the latter process can best be measured in an indirect manner, namely by the decomposition of a non-nitrogenous organic substance, such as glucose, added to the water, since the process of glucose decomposition was found to be controlled by the concentration of available nitrogen or by the rate of its liberation. It was thus calculated that about fifty per cent of the total organic matter in the water is readily attacked by the bacteria, under favorable conditions; of this decomposed organic matter, about sixty per cent is completely oxidized and about forty per cent converted into bacterial cell substance.

Among the factors influencing the rate and extent of decomposition of the organic matter in the water, the following were found to be of paramount importance: 1. The temperature of the water; the lower the temperature, the smaller is the amount of organic matter decomposed in a given period of time or the longer does it take to bring about the same degree of decomposition. When water taken in the neighborhood of Woods Hole was placed at definite temperatures, in

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closed containers, the amount of oxygen consumed in 6 days, per liter of water, was 0.26 cc. at 2° C., 0.83 cc. at 12°, 1.39 cc. at 22° and 1.14 cc. at 34° C. 2. Abundance of available organic matter. This was demonstrated by the fact that when water was filtered through different types of filters, which tended to remove some of the suspended organic matter, such as Seitz filters, the rate of decomposition of the organic matter, as measured by the amount of oxygen consumed in a given period of time, was considerably reduced. 3. The water mass itself, as modified by distance from bottom, by its relation to photosynthetic zone, by latitude, by movements, especially oceanic circulation and vertical mixing. In water taken from the Gulf of Maine, the greatest oxygen consumption took place at a depth of 40 meters, while at Georges Bank, with greater vertical circulation, in the water just above the bottom. 4. Concentration of available inorganic nutrients; the influence of available nitrogen upon the decomposition of carbohydrates has already been mentioned; certain preliminary investigations also point to the significance, in this process, of available phosphorus.

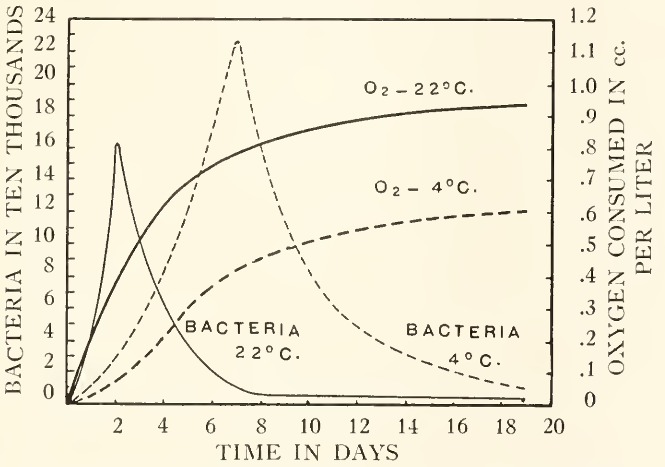

FIG. 1. Influence of temperature upon bacterial development and oxygen consumption in sea water.

Influence of Temperature

In order to illustrate further the effect of temperature upon the course of bacterial development and oxygen consumption, the results of a detailed and controlled experiment are reported in Fig. 1. The oxygen bottles were incubated under water. Although the maximum rate of bacterial development was reached as 22° C., within 2 days, and at 4° C., within 7 days, the consumption of oxygen continued even after that maximum was attained. The ratio between the periods of maximum bacterial development at the two temperatures was about 4 : 1. It is interesting to note that exactly the same amount of oxygen was consumed at both temperatures when this maximum was attained, namely 0.45 cc. per liter at 22° C. and 0.47 cc. at 4° C.

Influence of Organic Compounds

The second experiment illustrates the effect of two different forms of nitrogen upon the decomposition of a non-nitrogenous organic substance (glucose) added to sea water (Table I). In all cases, the water received 2 mgm. K_2HPO_4 per liter, to eliminate any effect due to a lack of available phosphorus in the water. The initial water contained 1,050 bacteria in 1 cc. and 5.43 cc. of oxygen per liter; the water was incubated at 22° C. In the decomposition of glucose added to sea water, the available nitrogen is definitely shown to be an important controlling factor; in the absence of available nitrogen, the addition of glucose did not result in any appreciable increase in oxygen consumption and bacterial development within three days, namely the time of most active bacterial multiplication in stored sea water; at a later period, however, active glucose decomposition sets in. However, there was still insufficient nitrogen available from the

TABLE I

Influence of nitrate and ammonia nitrogen upon the decomposition of glucose in sea water *

Glucose added	Source of nitrogen	Amount of nitrogen added	Bacteria in 1 cc.		Oxygen consumed	
			3 days	7 days	3 days	7 days
mgm. per liter		*mgm. per liter*			*cc. per liter*	*cc. per liter*
0	0	0	360,000	25,500	0.54	0.73
0	$NaNO_3$	0.1	345,000	17,000	0.52	0.69
0	$(NH_4)_2SO_4$	0.1	540,000	15,500	0.60	0.91
1.5	0	0	515,000	54,000	0.65	1.32
3.0	0	0	384,000	44,000	0.72	1.54
6.0	0	0	340,000	52,000	0.64	1.54
1.5	$NaNO_3$	0.1	1,930,000	42,500	0.95	1.27
3.0	$NaNO_3$	0.1	1,650,000	7,000	1.79	2.43
6.0	$NaNO_3$	0.2	3,200,000	7,200	2.74	3.69
1.5	$(NH_4)_2SO_4$	0.1	1,750,000	7,800	1.27	2.10
3.0	$(NH_4)_2SO_4$	0.1	1,880,000	6,000	1.76	2.37
6.0	$(NH_4)_2SO_4$	0.2	4,600,000	24,000	2.72	3.77

* Fresh water contained 1,050 bacteria in 1 cc. and 5.43 cc. oxygen per liter; bottles incubated at room temperature.

decomposition of the organic matter in the water, even after 7 days, to result in the oxidation of appreciable quantities of glucose; when 1.5 mgm. glucose was added per liter, the increase in oxygen consumption was 0.59 cc.; with another 1.5 mgm. glucose, the increase was only 0.22 cc., while the third portion of 1.5 mgm. glucose brought no further increase at all. It is interesting to note that the oxidation of glucose in 7 days resulted in an increase in oxygen consumption just about equivalent to that which took place in the water itself. The addition of 0.1 or 0.2 mgm. nitrogen to the water receiving the glucose greatly increased the rate of its decomposition. The two sources of nitrogen had about the same effect upon the rapidity of utilization of glucose by bacteria.

TABLE II

Oxygen consumption in the decomposition of amino acids in sea water by bacteria

Compound	Amount added	Oxygen consumed, cc. per liter			Theoretical oxygen required for complete oxidation of 1 mgm. of compound *	Bacteria in 1 cc.
		2 days	5 days	Per 1 mgm. of amino compound used		2 days
	mgm. per liter					
Water alone	—	0.71	1.01	—	—	820,000
Glycocoll	2	0.65	1.19	—	—	1,100,000
Glycocoll	4	1.42	1.95	0.38	0.64	2,050,000
Alanine	2	1.39	2.03	—	—	1,600,000
Alanine	4	2.03	2.94	0.46	1.08	3,700,000
α-Phenylalanine	2	2.08	2.77	—	—	950,000
α-Phenylalanine	4	3.66	4.95	1.09	1.93	1,200,000
Glutamic acid	2	1.31	1.78	—	—	1,950,000
Glutamic acid	4	2.30	3.24	0.73	0.98	3,550,000
Tyrosine	2	1.99	2.59	—	—	1,090,000
Tyrosine	4	3.57	4.58	1.00	1.68	1,600,000
Asparagine	2	0.82	1.23	—	—	1,120,000
Asparagine	4	1.32	1.97	0.37	0.73	2,100,000

* For complete oxidation of compound, assuming that nitrogen is left as ammonia.

The consumption of oxygen takes place not only at the expense of the carbohydrates in the water, but also in the decomposition and oxidation of the organic nitrogenous constituents, as brought out in Table II. Four amino acids, namely glycoçoll, *d*-alanine, α-phenylalanine, *d*-glutamic acid and tyrosine, and asparagine, were added to fresh sea water, in concentrations of two and four milligrams per liter. Consumption of oxygen and bacterial multiplication were determined, after two and five days incubation of the water, in the dark at 20° C.

For the calculation of the amount of oxygen consumed per unit of nitrogenous compound, the results obtained after five days incubation were used. The increase in oxidation for the addition of 4 milligrams of compound above the water receiving the 2-milligram portions was used as basis of interpretation of the results. This experiment brought out the fact that various simple nitrogenous compounds are readily oxidized by marine bacteria in sea water; a half or more of the theoretical amount of the different compounds was destroyed in 5 days, as measured by the amount of oxygen consumed; the rate of oxidation of the various compounds was found to vary with their specific chemical nature. These results tend to explain the rapid rate of decomposition of zoöplankton in sea water, as compared with that of the marine algæ (Waksman, Carey and Reuszer, 1933). Because of the greater proportion of available nitrogen in the former, their decomposition is accompanied by the liberation of nitrogen in the form of ammonia or as other simple compounds, while the decomposition of the second is accompanied by nitrogen consumption. The importance of the carbon-nitrogen ratio of the organic complex is evident. Substances with a narrow ratio will, on decomposition, result in a rapid liberation of the nitrogen. This nitrogen is partly utilized by the bacteria in the process of decomposition of the carbohydrates and is partly left in the water in a form available for the growth of the algal and diatom populations.

Table III

Influence of light upon bacterial development

Incubation	Oxygen, cc. per liter		Bacteria in 1 cc.	
Days	Dark	Light	Dark	Light
0	5.60	5.60	3,580	3,580
2	5.40	5.91	10,500	130,000
4	5.10	5.69	2,500	11,000
6	5.17	6.50	2,200	8,500
9	4.86	6.69	6,500	9,600
12	4.72	6.97	15,000	14,200

Relationship of Photosynthesis and Phytoplankton Development to Bacterial Activities in Sea Water

A parallel development of diatoms and bacteria in sea water has previously been reported (Gran, 1933, and Waksman et al, 1933). It is quite likely that this is due primarily to the fact that the bacteria

TABLE IV

Rapidity of bacterial decomposition of freshly synthesized plant substances

Incubation	Oxygen, cc. per liter			Bacteria in 1 cc.		
Days	Dark	Light	Light followed by dark for 5 days	Dark	Light	Light followed by dark for 5 days
0	5.41	5.41	—	10,000	10,000	—
3	4.95	5.67	5.04	28,000	17,000	14,500
6	4.79	5.52	?	9,000	19,500	?
10	4.55	5.84	5.43	7,100	6,000	9,500
20	4.47	6.96	5.91	20,500	13,000	18,300
30	4.30	7.60	—	17,500	9,000	—

feed either upon the organic substances secreted by the diatoms or upon those cells which, because of mechanical disintegration, become subject to bacterial attack. Fresh sea water was placed in oxygen bottles; some of these were incubated in the darkness at room temperature and under water, and some were exposed to the light, also under water. At definite intervals, some of the bottles were removed and analyzed for oxygen and bacterial numbers (Table III). The results show that continued decomposition of the organic matter took place in the darkness, while, in the light, organic synthesis predominated, as shown by the liberation of oxygen; the newly synthesized material supported a more abundant bacterial population.

TABLE V

Synthesis and decomposition of organic matter in sea water

Incubation, days		Oxygen, cc. per liter			Bacteria in 1 cc.		
Harbor	Laboratory	Dark	Light	Light followed by dark	Dark	Light	Light followed by dark
0*	—	5.80	5.80	—	—	—	—
7	7	4.60	6.19	5.01	—	—	—
7	14	—	—	4.05	—	—	—
0†	—	5.37	5.37	—	—	—	—
7	9	4.59	6.11	5.01	2,000	14,630	46,900
7	18	—	—	4.11	—	—	17,250

* Experiment begun on July 27.
† Experiment begun on August 10.

In order to demonstrate to what extent this freshly synthesized organic matter can be decomposed by the bacteria in the water, the above experiment was repeated. Some of the bottles, after exposure to light for varying periods of time, were incubated, for a period of 5 days, in the darkness (Table IV). The synthesized organic substances are found to be readily subject to bacterial decomposition, as shown by the rapid reduction in the oxygen, when photosynthesis is excluded.

The results of two other experiments are reported in Table V. Sea water was placed in light and dark bottles and incubated directly in the harbor, about five feet below the surface; a modified procedure of Winberg (1934) was employed. Here as well, some of the bottles, which have been exposed to light in the harbor for a period of 7 days were later incubated in the darkness, for varying periods of time. The temperature of the outside water was about 15° C. and of the tank in laboratory, about 20° C. The newly synthesized organic matter was again found to be rapidly decomposed, as shown by the disappearance of the oxygen and by the increase in bacterial multiplication, when photosynthesis was stopped.

TABLE VI

Decomposition of organic matter in water from Gulf of Maine and Georges Bank, in the spring *

Station number	Depth	Temperature of water	Bacteria in 1 cc. of water	Water incubated for 5 days at room temperature			
				Water alone		Water and glucose †	
				Oxygen consumed	Bacteria in 1 cc.	Oxygen consumed	Bacteria in 1 cc.
	meters	° C.		*cc. per liter*		*cc. per liter*	
2441	5	7.5	—	1.28	621,000	3.68	1,375,000
2441	50	4.3	—	0.79	77,500	1.44	99,000
2441	225	5.8	—	0.71	24,000	1.30	—
2442	5	7.0	55	0.88	200,000	1.08	164,000
2442	50	3.9	8.5	0.66	48,300	1.46	714,000
2442	225	5.6	1	0.48	79,500	0.57	79,000
2443	5	6.9	2	1.00	27,400	1.69	71,000
2443	25	7.9	7	0.77	36,000	1.11	36,000
2444	5	6.35	19	0.98	96,000	1.13	101,500
2444	33	6.44	8	0.52	18,000	0.97	85,500

* Samples taken on May 20, 1935.

† Two mgm. per liter.

Decomposition of Organic Matter in Sea Water at Different Seasons of Year and at Different Latitudes

An attempt can now be made to interpret the results obtained on the decomposition of the organic matter in sea water taken from a group of stations, at different depths, and at different seasons of the year. Water taken in the spring of the year from both deep (Gulf of Maine) and shallow (Georges Bank) stations showed the largest amount of rapidly decomposing organic matter at the surface, gradually decreasing with depth (Table VI). The amount of oxygen consumed in the presence of glucose was, on the average, just about twice as high as in the water alone; this would tend to confirm the previous conclusions concerning the liberation of an equivalent amount of nitrogen in an available form, in the decomposition of organic matter in sea water. Water taken from the Gulf of Maine stations, in the late summer and fall, showed an increase in readily decomposable organic matter with increasing depth, then decreasing again in the water nearer to the bottom; the same was true also of the water taken in the Gulf of Maine stations during the following summer (Table VII).

TABLE VII

Bacterial activities in the water of the Gulf of Maine in summer and fall

Date of sampling of water	Depth	Water incubated 5 days at room temperature	
		Oxygen consumed	Bacteria in 1 cc.
	meters	*cc. per liter*	
August 5, 1934	5	0.86	—
August 5, 1934	40	1.26	—
August 5, 1934	229	1.45	—
November 22, 1934	5	0.43	34,000
November 22, 1934	40	0.90	102,500
November 22, 1934	236	0.70	244,000
November 22, 1934	5	0.64	130,000
November 22, 1934	40	0.72	140,500
November 22, 1934	213	0.59	211,500
July 8, 1935 *	0	0.42	225,000
July 8, 1935	50	1.02	182,300
July 8, 1935	125	0.67	214,000
July 8, 1935	0	0.13	373,000
July 8, 1935	50	0.77	339,000
July 8, 1935	200	0.35	191,000

* Incubation of water, 3 days.

Several stations were taken, during the winter of 1935, from Cape Cod Light to Bermuda; the rate of oxygen consumption and bacterial multiplication in the water from those stations, kept in closed bottles at a given temperature (18–22° C.) and for a certain period of time (5 days), was determined. The water was filtered through sterile sand filters, so as to eliminate the interference of coarser material. The amount of oxygen consumed was reduced appreciably by the filtration process, namely, in one test from 0.70 to 0.35 cc. per liter and in another from 1.26 to 0.74; this was due to the fact that the readily decomposable larger diatoms, protozoans and other forms of life were removed. The most striking result of these experiments (Table VIII) is the poverty of the waters close to Bermuda. Although sufficient bacteria were found in the water and especially in the bottom material (174,000 bacteria in 1 gram of fresh material), there was not sufficient organic matter to support active bacterial development.

TABLE VIII

Oxygen consumption in water taken from a series of stations from Cape Cod to Bermuda

Station number	Position	Depth	Temperature	Oxygen consumed*	Bacteria in 1 cc.	
					Start	5 days
		meters	° C.	*cc. per liter*		
2308	42° 21′ × 69° 34′	5	5.2	0.74	414	550,000
2309	42° 32′ × 69° 13′	5	5.2	1.00	614	213,750
2310	41° 27′ × 69° 33′	5	4.3	0.92	83	65,800
2311	41° 18′ × 69° 21′	5	4.5	0.96	68	132,000
2312	40° 12′ × 68° 21′	5	9.4	—	141	215,000
2312	40° 12′ × 68° 21′	50	5.4	0.83	399	207,500
2312	40° 12′ × 68° 21′	190	3.4	0.95	3,000	375,000
2313	38° 25′ × 68° 18′	5	11.8	0.63	52	169,000
2314	38° 20′ × 68° 17′	5	20.0	0.78	62	347,000
2315	37° 42′ × 68° 02′	5	21.5	0.62	412	204,000
2316	36° 09′ × 67° 30′	5	18.5	0.75	52	78,200
2317	33° 56′ × 65° 52′	5	19.1	0.10	66	79,000
Grog's Harbor, Bermuda		1–6	17.0	0.24	78	16,500

* Five days incubation at room temperature.

Influence of Oxygen Tension

The concentration of oxygen in the water was previously found (Waksman and Carey, 1935*a*) to have an important influence upon bacterial activities and upon oxygen consumption, when the water was allowed to incubate. Those results were obtained by the use of Seitz-filtered water. It was found in subsequent studies that this filtration process removes a large part of the suspended and readily decomposable organic matter in the water, thus modifying considerably the extent of decomposition of the organic matter. This is illustrated in Table IX. In the unfiltered water, the organic matter decomposed readily even at the lowest concentration of oxygen; in the Seitz-filtered water, however, the amount of organic matter decomposed with a low oxygen content was much less. With an increase in the concentration of oxygen in the water, the rate of organic matter decomposition, although remaining less in the filtered water, approaches that of the unfiltered water. These results tend to establish certain facts: (*a*) the small amount of organic matter removed by filtration through Seitz filters undergoes rapid decomposition; (*b*) the oxygen tension required for the decomposition of the organic matter passing through the filter is greater; (*c*) the type of the bacteria developing on the organic matter in the Seitz-filtered water is different, as shown by the fact that much larger numbers are produced accompanied by a much smaller amount of oxygen consumed. The difference in the numbers of bacteria given by filtered and unfiltered water may point to radical differences in the chemical nature of the two fractions of the organic matter in the water. This confirms some observations previously reported (Waksman and Carey, 1935*a*).

TABLE IX

Influence of oxygen tension in unfiltered and Seitz-filtered sea water upon bacterial activities

Treatment of water	Unfiltered water				Seitz-filtered water			
Oxygen content at start, cc. per liter	0.57	2.66	4.97	5.28	1.14	2.38	2.89	4.94
Oxygen content, after 8 days	0.05	2.16	4.24	4.61	0.89	2.07	2.50	4.40
Oxygen consumed	0.52	0.50	0.73	0.67	0.25	0.31	0.39	0.54
Bacteria in 1 cc., after 8 days	11,500	7,000	2,150	1,300	9,000	230,000	122,000	102,000

Continued Decomposition of Organic Matter in Stored Water

In all previous experiments, only short incubation periods were employed. The question naturally arises as to what happens when prolonged periods of incubation are used. In order to determine this effect, three large carboys were carefully washed and filled with sea water as follows: (*a*) fresh water from Woods Hole Harbor, (*b*) same water filtered through a column of washed sterile sand, (*c*) fresh water from Gulf of Maine. Once a week, 1,200-cc. portions of water were removed and placed in oxygen bottles; the amount of oxygen absorbed in 7 days, by incubating the water in the dark, was then determined. The carboys were well shaken every day so as to increase the oxygen content of the water. The results (Fig. 2) show that the unfiltered harbor water gave the greatest amount of decomposition and that the rate of decomposition of the organic matter in the water was gradually reduced. The total amount decomposed in 6 weeks per liter of water, in terms of oxygen consumed, was 1.64 for the unfiltered harbor water, 1.19 for the filtered harbor water and 1.24 for the Gulf of Maine water.

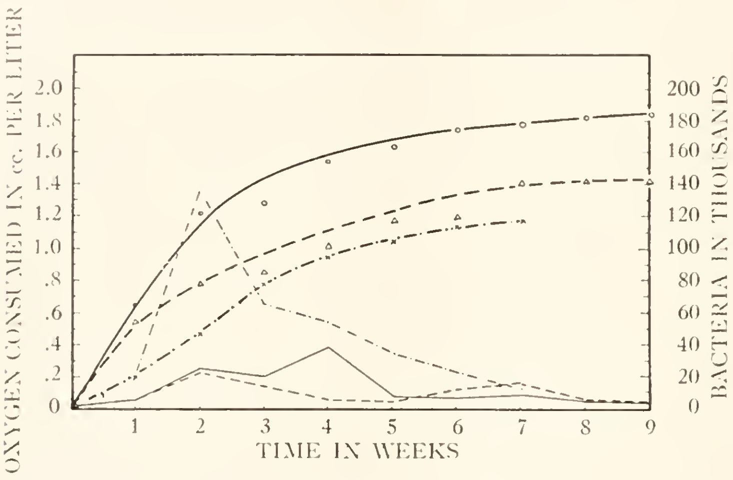

FIG. 2. Changes in bacterial numbers and oxygen consumption upon continued decomposition of organic matter in sea water. Heavy lines represent oxygen consumption; light lines, bacterial numbers. Heavy unbroken line, curve for unfiltered water from Woods Hole; heavy broken line, curve for filtered water from Woods Hole; ·—· represents curve for unfiltered water from Gulf of Maine.

SUMMARY

1. Investigations are reported on the decomposition of organic matter in sea water placed under controlled laboratory conditions. It has been found that, under these conditions, the organic matter in the water, both in suspension and in solution, can undergo rapid decomposition. It is quite possible that a change in temperature, resulting from the sudden warming up of the water, may bring about not only an increase in the biological reactions, but also a change in their very nature; this limitation need not invalidate the results obtained, since these results are not a measure of what actually

happens in nature under different conditions, but what may happen under any one set of conditions.

2. The abundance of readily decomposable organic matter in sea water was measured by the rate of oxygen absorption in the water, incubated under uniform conditions. The rapidity of liberation of nitrogen in the decomposition of the organic matter can be measured by the rate of decomposition of glucose added to the water.

3. The amount of organic matter in sea water readily attacked by bacteria varies with the rate of production of the organic matter, with photosynthesis and with the water mass; at the surface of the water, it falls off as one goes southward.

The authors are indebted to Mr. T. C. Cordon, for assistance in making the oxygen determinations.

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THE KINETOCHORE OR SPINDLE FIBRE LOCUS IN AMPHIUMA TRIDACTYLUM

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In another place I have expressed the conviction that the mitotic movement of chromosomes is closely associated or perhaps even dependent on the activities of the kinetochore (Schrader, 1935). In taking such a position I of course make no claim to originality, for the belief that the kinetochore is of the utmost importance in mitosis has become rather general in recent years, even though it is one that is more often tacitly assumed than openly stated. Indeed Metzner (1894), the first investigator to describe these bodies, already had a very definite conception of their function, as his name "Leitkörperchen" signifies. That later investigators have invented terms of their own may in some cases have been due to ignorance of Metzner's work, but in others it was due to hesitation to ascribe to the structure in question, without further evidence, the rôle that his term implies. Thus we have beside Leitkörperchen, spindle fibre attachment (many geneticists), Insertionsstelle (Belar and others), attachment body (Schrader), spindle fibre locus (Metz), commissure (Lorbeer), kinetochore (Sharp), and many more.

If my own term, attachment body, merits justification I can only say that I used it because I considered it a descriptive term which does not carry the implication of function.[1] It merely denotes that special region in the chromosome to which the spindle fibre or half-spindle component is connected—as seen in fixed preparations. However, questions of terminology interest me so little that I am quite willing to abandon this nomenclature and accept the term kinetochore, which, even though it ascribes to this body a dynamic rôle of which we have no final proof, is a name that has been used by Sharp (1934) in a book of wide distribution. It thus bids fair to become more generally adopted (as it already has by several other botanists) and therefore to do away with the present unprofitable confusion.

Perhaps my acceptance of Sharp's term is made easier by my belief that, even though no conclusive evidence for it is available, the kinetochore is indeed intimately concerned with certain dynamic processes of the chromosome. Such as they are, the foundations of this belief may be stated quite simply. They are that if a kinetochore is visible, it definitely serves as the chromosomal base for the connection between the chromosome and the pole. Further, in following the chromosome through a cell cycle, the kinetochore can be demonstrated cytologically before there is any trace of the half-spindle component (i.e. the connection between chromosome and pole). Therefore even though an important rôle in the mitotic movement of the chromosome be ascribed to the latter, it is not unfair to assume that the kinetochore may be causally involved. As a third support for the belief in a kinetic rôle may be regarded the fact that in the progress from the equator to the pole it is the kinetochore region of the chromosome that leads the way. (But it should not be forgotten that the cases of *Sciara* (Metz, 1933) and *Micromalthus* (Scott, 1936) provide notable exceptions to this rule.) Finally, it seems definitely established in at least some forms that the loss of the kinetochore (be it a natural or experimental effect) results in the omission of the affected chromosome from the division figure and brings about its elimination from the process of orderly mitotic distribution.

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[1] Professor C. W. Metz, of the Carnegie Institution, and I have spent a not inconsiderable amount of time in discussing this terminology. His term, *spindle fibre locus*, represents an attempt to preserve a noncommittal attitude concerning the function of the structure involved.

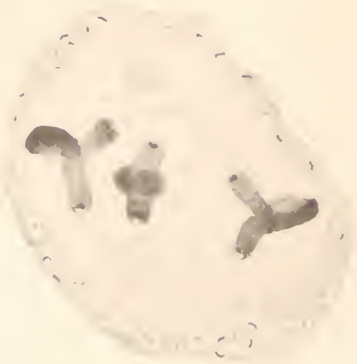
In brief, the evidence is not final, but it serves as an adequate basis for a study such as the present paper represents.

METHODS

The investigation was made principally on the testes of the amphibian *Amphiuma tridactylum.* The decisive observations were all made on material fixed in Champy's or Benda's fluid, the latter prepared with a very small amount of acetic acid (not exceeding one drop). Other fixations served only a comparative purpose. Heidenhain's hæmatoxylin, strongly extracted, proved the best method of staining for detailed study, but certain of my conclusions, as will appear, involve such mitochondrial stains as those of Benda and Kull. Sections were usually cut as thin as 4 μ, which unfortunately leaves few of the nuclei entire but is advantageous for detailed study. Through Professor E. B. Wilson's kindness I was enabled to study his beautiful slides of the arachnid *Opisthacanthus elatus*, my findings confirming certain of his observations on that animal which pertain to my present work.

FIRST SPERMATOCYTE

In the prophase period, the kinetochore cannot be demonstrated with certainty until the late diplotene and early stages of diakinesis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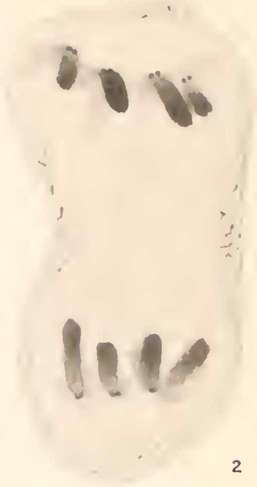
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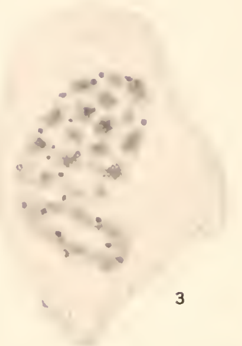
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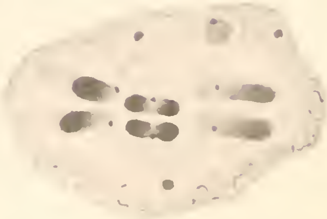
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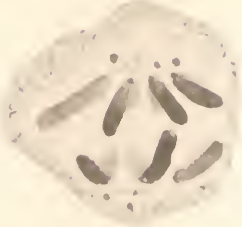
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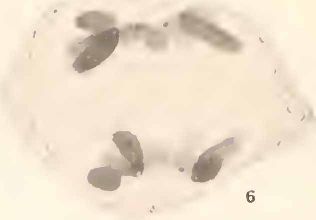
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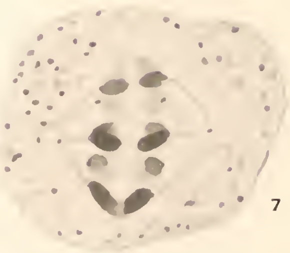
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PLATE I

have been reached. At that time hæmatoxylin preparations, in which the chromosomes have been destained so as to appear an even gray, show the kinetochore as a darker body, not very sharply outlined (Figs. 8, 9, 10). In *Amphiuma*, therefore, the kinetochore at this time shows little or no trace of the achromatic properties that characterize it in many other forms (McClintock, 1933).

In the pachytene and diplotene stages preceding this I have been unable to make a definite identification of the kinetochore, but it is only fair to say that the chromosomes at these phases destain rather unevenly (heterochromatin?) and that it may be a technical difficulty of this sort that is here at fault. Further work involving other techniques is evidently called for.

In the early stage of diakinesis already mentioned, two kinetochores are present in each tetrad. It is to be noted that these are quite independent of each other and that each is associated with a different pair of chromatids (Figs. 8, 9, 10). But though the extreme degree of destaining employed in my technique allows of no detailed analysis of these chromatids, the components of a given pair can be traced in several cases and in none does the line of demarkation between them extend to the kinetochore (Fig. 8). The latter is undivided though it is associated in every instance with two chromatids.

Shortly after this period and at a time when the rapidly condensing, diakinetic tetrads are no longer so closely associated with the nuclear periphery, each of the two kinetochores carried by a tetrad shows a double structure. The two bodies that then characterize each kinetochore have the form of short rods, rather sharply outlined, whose long axis is parallel to the long axis of the chromatids (Figs. 11

PLATE I

Drawings No. 1 to No. 6 made with Zeiss 2 mm. apochromatic objective (n. ap. 1.4), and 10 × ocular. No. 7 to No. 20, inclusive, with 15 × ocular.

1. First spermatocyte metaphase. Not all tetrads in section are shown. Benda fixation and Kull stain. Chromosomes yellow, commissural cup colorless, spindel spherules red, chondriosomes bluish red.

2. Late anaphase of first spermatocyte, with two spindle spherules in each dyad. Benda fixation and hæmatoxylin stain.

3. Interkinesis showing granules. Benda—hæmatoxylin.

4. Early anaphase of second spermatocyte. Separating chromosomes showing that here the kinetochores do not lead the way to the poles. Only one spindle spherule in each daughter chromosome. Benda—hæmatoxylin.

5. Late anaphase of second spermatocyte. Kinetochore now leading poleward movement. Extraneous granules near the poles. Benda—hæmatoxylin.

6. Second spermatocyte division, telophase. Benda—hæmatoxylin.

7. Spermatogonial anaphase in the mouse. Champy fixation, Kull stain. Chromosomes brown, kinetochores and centrioles red, chondriosomes bluish red. Preparation made by A. G. Steinberg.

and 12). Nevertheless, the region of the kinetochore as a whole does not appear to be split, a fact which has some bearing on the analysis of its structure.

With further condensation of the chromatids, these tiny rods seem to shorten until in the fully condensed tetrad they appear as granules or tiny spheres (Figs. 13–15). It is during this process that a light or non-staining region comes to surround them, and I believe that it is this latter area that is the most conspicuous portion of the kinetochore in most other organisms. But whereas it is usually described quite correctly as an achromatic band or commissure that extends transversely through the chromosome, it appears in the *Amphiuma* tetrad more as a notch. Whether this form assumed in *Amphiuma* is in part the result of the bending and torsion of the tetrad I am unable to say, but it seems likely that the explanation is not so simple. Certainly the so-called secondary constrictions, which also appear as transverse achromatic bands, can in the case of *Amphiuma* be traced from an early prophase to final condensation of tetrads, and there is no such alteration in form as that described for the kinetochore (Fig. 15).

Indeed, a very careful examination of well-fixed material indicates that, in the condensed tetrad at least, the form of the achromatic portion of the kinetochore is not so much a notch as a rounded or cup-shaped depression directing its open side toward the pole. The tiny, intensely staining spherules already mentioned adhere to the inner side of this cup, close to its bottom (Figs. 13, 14, 15).

The relation of the kinetochore to the mitotic spindle is quite clearly shown in some of my preparations. White (1935) has recently advanced some evidence convincing him that it is to the commissural or achromatic portion of the kinetochore that the spindle fibre (i.e. the half-spindle component) attaches. But as an examination of my Figure 18 will show, this is not the case in *Amphiuma*. It is from the tiny chromatic spherule that the half-spindle component takes its origin and not from either the commissure or the kinetochore as a whole. It follows therefore that where tetrads are concerned, each half-spindle component has a double source, since two spindle spherules are carried in each commissural cup. That these two, separately arising spindle elements are themselves composed of finer fibrillæ and that they usually join to make a single half-spindle component before reaching the pole is implicit in what I have reported elsewhere (Schrader, 1935). But though the spherules are clearly more directly involved in the association with the spindle, it is equally certain that the commissural cup in which they rest forms an integral part of the whole apparatus and that it is the commissural cup and the spindle spherules together that constitute the kinetochore.

It is further evident that each of the spindle spherules is associated with a chromatid. The complete tetrad as shown in Figs. 1 and 15 has four spindle spherules, two directed to each pole. Views in which one chromatid is superimposed on the other likewise show superimposition of spindle spherules, as appears from several of my figures. It is only when both chromatids are in face views that the two spindle spherules of a given pair appear as distinct and separate as they actually are.

The further history of the first spermatocyte shows no more than what might be expected from the above. In the actual division, the kinetochore leads the way toward the pole and the chromosome regions farthest removed from the kinetochore are the last to leave the equator. In late anaphases and in the telophase it becomes difficult to make out the detailed structure of the kinetochore, but the two spindle spherules associated with each half of the tetrad continue to stand out sharply until the interkinetic phase is initiated (Figs. 1, 2 and 3).

INTERKINESIS

The period between the first and second spermatocyte divisions is an aggravatingly difficult one. As the telophase chromosomes become diffuse, there appear many darkly stained granules in the nuclear area (Fig. 3). A little later, when the new nuclear membrane has formed, most of these are peripherally placed. At first I worked on the assumption that these granules are nothing more than spindle spherules which temporarily sever their connection with the chromosomes during this period and become enlarged. But in spite of the wealth of my material, I was unable to make a seriation of stages that might be involved in such a process. The difficulty arises in several ways. Thus certain granules quite extraneous to the chromosomes are present in many telophases of both divisions (Figs. 4 and 5) and these are almost certainly included in the nucleus. Since they vary in number, a mere count is therefore useless. Nor can these last named granules be differentiated from the others on the basis of dimension, for size differences are too small and variable to be of any use. Since my differential stains did not serve to make a distinction either, the hypothesis was temporarily abandoned. It is quite possible that these granules have nothing to do with the kinetochores and that the latter cannot be seen during the interkinesis.

The Second Spermatocyte

The kinetochores of the second division can be definitely recognized shortly before final condensation of the chromosomes. At this time the two chromatids are already quite clearly demarked from each other. Each spindle spherule lies in a light area which in form approximates the typical commissure more closely than does the corresponding region of the first spermatocytes. Side views, however, still show the notch-like configuration (Fig. 20). But although the total number of spindle spherules has not changed between the first spermatocyte telophase and the second spermatocyte metaphase, an essential structural difference is to be observed in the fact that, instead of one kinetochore with two spindle spherules (Fig. 2), we now have two kinetochores each with a single spherule (Fig. 4). In short, the division line between the chromatids now quite definitely extends through the kinetochore. But conditions for detailed study are not as favorable as in the first division and nothing further can be said concerning the detailed structure of the kinetochore at this time.

In the initial chromosome movement toward the pole, the kinetochore does not lead the way as in the first division. Instead, the distal ends of the chromosome move just as rapidly as the kinetochore region, so that it is evident that we are here concerned with what has

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Plate II

All drawings except No. 16 made from preparations fixed with Benda and stained with hæmatoxylin.

8. Tetrad in early diakinesis showing kinetochore as a rather indefinite, dark region.

9 and 10. Slightly later than preceding. Kinetochore becoming more definitely outlined.

11 and 12. Middle phases of diakinesis. Each kinetochore showing two spindle spherules elongated in the long axis of the chromosome. Commissural region beginning to appear as a lighter area.

13. Late diakinesis showing two commissural cups, each with two spindle spherules (one pair superimposed).

14. Metaphase tetrad, one arm cut.

15. Tetrad in metaphase or early anaphase, showing commissural cups, spindle spherules and one secondary constriction.

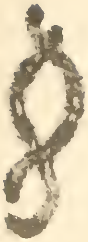
16. Tetrads in early anaphase, stained with Kull. Kinetochore appearing as red, hemispherical region below a shallow depression in the yellow chromosome. Further differentiation destains commissural part of the kinetochore and leaves spindle spherules as red bodies.

17. Tetrad in early anaphase. Only one kinetochore shown.

18. Dyad in anaphase of first spermatocyte, showing relation of half-spindle component to the two spindle spherules.

19. Polar view of dyad in metaphase of second spermatocyte division.

20. Side view of dyad in second spermatocyte. Each chromatid with one spindle spherule.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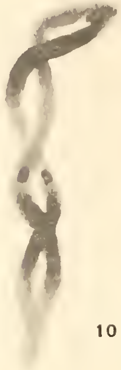
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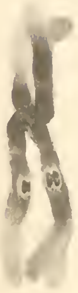
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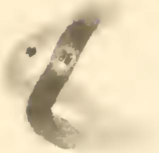
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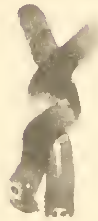
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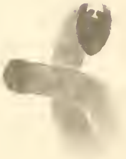
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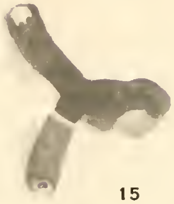
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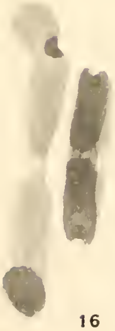
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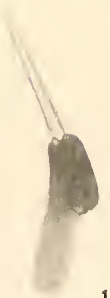
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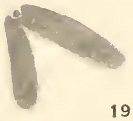
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PLATE II

been so often interpreted as a mutual repulsion of sister chromatids (Fig. 4). It is only toward the end of the anaphase movement that the kinetochore once more takes the lead, indicating that the force of repulsion becomes less effective as the distance between the chromatids increases (Fig. 5). It is more than likely that the kinetochore functions similarly in both spermatocyte divisions and that the observed differences in the anaphase movement arise from the special conditions presented by the tetrad formation of the first division.

In any case, the result of the second division is a telophase in which each chromosome carries one kinetochore with a single spindle spherule (Fig. 6). As this telophase group enters the diffuse condition prior to sperm formation, granules, such as are encountered in the interkinesis, once more appear.

Somatic and Spermatogonial Divisions

My material for an investigation of these stages is very limited. Apparently the behavior of the kinetochore parallels that observed in the second spermatocyte division. At metaphase, when the conditions are most favorable for study, the line of division in the chromosome is already present, and each half is provided with its own kinetochore. This means, of course, that division of the latter must take place during the prophase at the latest.

Relation to Chromosome Structure

There seems to be a general tendency to regard the kinetochore simply as a chromomere that has undergone certain temporary changes. That view has been expressed recently by Lorbeer (1934) who says: "There are examples from which it appears clearly that the kinetochore (his commissure) is neither linin nor matrix, but a chromomere exactly like any other chromomere—." The difficulty that I have experienced in tracing the kinetochore through resting and prophases lends some support to such a view, for in many forms the ordinary chromomeres are likewise difficult to follow through these stages.

Nevertheless, I make bold to state that, if this is the origin of the kinetochore, the changes that it has undergone are both extensive and fundamental. Thus the ordinary chromomere has no structure homologous to the commissural cup and the spindle spherule. Again, even hæmatoxylin staining shows that there are considerable differences in the staining reactions, for the spindle spherules remain intensely colored after the rest of the chromosome structure has become decolorized.

But the most striking evidence for a special nature of the kinetochore lies in its response to certain mitochondrial stains. After staining by the Benda method, the chromosomes are brown or amber colored, whereas the kinetochore is violet. After Kull, chromosomes can be brought out as yellow, while the kinetochore region is red or pink. Nor is this red similar to that which characterizes the chondriosomes in the same cell, for the latter have a distinctly more purple cast (Fig. 16). This red region seems to underlie the superficial depression in the chromosome that characterizes the kinetochore and affords additional evidence that the latter is not merely a superficial structure.

A similar staining reaction has been reported already by Metzner (1894) in the salamander. To be sure, by no means all the bodies that he recognized as kinetochores were correctly identified, but his pictures leave little doubt that at least in some cases he was not in error. It is therefore plain that in spite of its ordinarily achromatic properties, the commissural region of the kinetochore will respond to certain special methods of dyeing.

With the Kull stain especially it is possible further to differentiate the kinetochore so as to decolorize the commissural cup entirely, leaving the spindle spherule a dark or intense red (Fig. 1). The result is a striking one, although the optical definition for detailed study is not as good as in a hæmatoxylin stain. But not a doubt can obtain that these staining reactions mark the kinetochore as a highly specialized structure and that the spindle spherule has a decidedly more pronounced reaction to the fuchsin of the Kull stain than has the general commissural substance.

Such staining is encountered not only in *Amphiuma*, but also in the spermatocytes of at least two widely different animals. One of these is the arachnid *Opisthacanthus elatus*, in which Wilson (1931) reported that after the Benda stain the kinetochore (his polar granule) appears a clear violet on the brown background of the chromosome. This is an observation that I can fully confirm and Wilson's drawing (his Fig. 29) shows the conditions perfectly.

The other animal is the common mouse (*Mus musculus*), in which the Kull stain brings out the kinetochore as a red body while the chromosome is yellow (Fig. 7). The staining reaction in this case is not a difficult one, and several of my students have been successful in obtaining it (A. Greenberg, M. H. Slud, A. G. Steinberg, among others).

It is, however, to be admitted that the size of the chromosome as well as the kinetochore in both *Opisthacanthus* and *Mus* are such that a detailed study comparable to that made in *Amphiuma* becomes difficult or even impossible. Nevertheless, they serve to show that my conclusions concerning the nature of the kinetochore in *Amphiuma* probably have a broader application. Whether it is always of the form that I have described is another matter. Thus it may be pointed out that Sharp (1929), Nebel (1935), and White (1935), among others, picture the spindle spherule as terminating a fine thread. Nebel states, however, that such a form is probably the result of acid fixation and that as the acid in the fixing fluid is decreased, the thread becomes shorter and finally disappears. I attempted to test this conclusion, but though in a sense my own findings support Nebel (since in them the absence of a thread is correlated with little or no acid in the fixing fluid), I was unable to obtain preparations in which the spindle spherule shows any thread. Indeed, after strongly acid fluids my material did not show the kinetochore at all.

THE KINETOCHORE IN THE MITOTIC CYCLE

It will be noticed in my drawings of *Amphiuma* that after extreme differentiation the centriole is so small as to be difficult to observe. I have found this to be the case even after the Benda stain, which in my experience brings out the centriole better than any other. In *Opisthacanthus*, on the other hand, the centriole is quite conspicuous after this stain, taking on a violet color that is much closer to that of the kinetochore than of the chondriosomes. Even without the rest of my evidence, these reactions would prevent me from proposing a chondriosomal nature for the kinetochore.

On the other hand, a relationship between the centriole and the kinetochore might well be considered. To be sure, the staining reaction just referred to offers but a fragile basis, but some additional slight evidence may be derived from certain observations of Belar (1929). He found that in the live chromosomes of *Chorthippus* the attachment region (the kinetochore) of the chromosome appears as a clear spot, and I can affirm this for another species of grasshopper. Unfortunately opportunity was lacking to study the living cells of *Amphiuma* with this point in mind. Now, a clear or glassy character has also been reported for the living centrioles in at least two forms. These are *Œcanthus* (Johnson, 1931) and *Drosophila melanogaster* (Huettner and Rabinowitz, 1933), and again I can confirm the observation, this time on material prepared by these workers themselves. However weak, the evidence is therefore not against the hypothesis of some sort of a relationship between centriole and kinetochore.

If there is such a relationship it may well be that in it lies the key to the puzzle of mitotic movement. But with so little evidence I have no intention of entering this old battlefield of hypotheses. I mention it chiefly in order to state that the idea of such a relation underlies the conclusions of some of my fellow workers, whether or not they are conscious of it. I am referring especially to certain convictions concerning the bouquet stage in the meiotic prophase, where, it will be remembered, the leptotene threads show a definite orientation toward a point on the nuclear periphery usually close to the centriole. It is very natural to conclude that this movement must be attributable to some type of rapport between centriole and kinetochores, and such an assumption is implicit in the writings of several investigators, notably Janssens (1924).

Confessing to a certain amount of sympathy with the view, I am nevertheless constrained to point out that there is some weighty evidence to the contrary. This derives from the very careful work of such investigators as Gelei (1921) and Mohr (1916). Mohr, for instance, has shown conclusively that in the leptotene polarization of the long chromosomes of *Locusta viridissima*, where the kinetochore must be close to median in position, it is the ends that are drawn toward the centriole. In fact the kinetochore region is in this phase as far as possible removed from the point of centralization.

By this same token it becomes questionable whether the polar granule of Pinney (1908) and Wenrich (1916) is to be regarded as identical with the kinetochore. Wenrich himself states (p. 64) that the polar granule appears at the same end of each chromosome to which the spindle fibre attaches, but he by no means commits himself to the view that it is the polar granule that is instrumental in this process of attachment. Indeed, he could hardly do so when it is considered that within a few sentences of the above he notes his agreement with the findings of Miss Pinney (1908) that in certain chromosomes similar granules also occur at the end of the chromosome opposite that to which the spindle fibre attaches. On page 69 he declares that the chromosome pair B "is characterized by the presence of large and well-marked polar granules at both ends." Similarly, although the "granules proximaux" that Janssens (1924) describes in the metaphase chromosomes are certainly kinetochores, it is by no means certain that these are identical with the granules to which he gives the same name in the bouquet stage. In view of the evident importance of these bodies a cautious attitude seems warranted.

Cytogenetic Aspects

That the kinetochore plays an important rôle in the cell cycle is sufficiently clear from its effects on crossing-over and chiasma formation. In Darlington's hypothesis (1932) concerning the meiotic mechanism it is the kinetochore that is accountable for the fact that division of the tetrads always occurs into pairs of chromatids. Such effective singleness of the kinetochore also would support Darlington's chiasma type and terminalization hypotheses "which demand that the identical chromatids should remain together at the spindle attachment, although nowhere else."

Nebel has recently (1935*a*) questioned this rôle that Darlington ascribes to the kinetochore. His doubts stem from his discovery that in the first meiotic anaphase of *Tradescantia reflexa* each pair of chromatids (in the sense of Darlington) is provided with four Zugfasern where the presence of even two would provide a definite objection to Darlington's hypothesis.

Now, "Zugfasern" (Belar's traction fibres) are regarded by Nebel as an attenuated form of the kinetochore, which is assumed during meta- and anaphase. Hence the presence of two or more Zugfasern indicates that the kinetochore has divided and therefore Darlington's singleness of that body does not hold.

In this connection I should like to point out again that the evidence for *Amphiuma* strongly indicates that the kinetochore is a compound body. In *Amphiuma* also, the first anaphase shows two spindle spherules for every pair of chromatids, but it is clear that these are not free to move independently of each other. They are held together by the undivided commissural cup. Since I regard Nebel's Zugfasern as equivalent to my spindle spherules, I do not consider that his demonstration of their division and subdivision (which I do not question for one moment) carries with it the proof that the kinetochore as a whole has also divided. In short, Darlington's thesis does not necessarily collapse in the face of Nebel's fine evidence.

On the other hand, Nebel's implication that at metaphase each chromatid (or chromonema) is provided with a spindle spherule is well substantiated by my findings. And further, the presence of four spindle spherules for each *Amphiuma* tetrad, whereas eight are to be encountered in the tetrad of *Tradescantia*, can therefore only support Nebel's conclusion (1935*b*) that there is a definite difference between animals and plants with respect to the number of chromonemata involved during meiosis (Nebel reports four for the orthopteran *Dissosteira* and eight for *Tradescantia*).

SUMMARY

1. The kinetochore is a compound body composed of a commissural region and a spindle spherule, so far as univalent chromosomes are concerned.

2. The half spindle component connects with the chromosome through the spindle spherule.

3. Already during the diakinetic phases of meiosis, each tetrad has two kinetochores which are distinct and separate from each other. Each of these is provided with two spindle spherules, so that each tetrad carries a total of four spindle spherules.

4. In the first spermatocyte division, the kinetochores lead the way to the poles. The dyads resulting from this division each show two spindle spherules until the resting stage is reached.

5. At metaphase of the second spermatocyte, each dyad is already sharply differentiated into two chromatids or univalent chromosomes. Each of these carries a kinetochore provided with a spindle spherule.

6. Aside from its structure, various staining reactions mark the kinetochore as an organ quite distinct from the ordinary chromomere.

7. The possible relation of the kinetochore to cell mechanics is discussed, and certain cytogenetic aspects are considered.

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